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TITLE: Triterpene compositions and methods for use thereof

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☑ 14. Document ID: US 6689398 B2 Relevance Rank: 88

L4: Entry 50 of 82 File: USPT Feb 10, 2004

DOCUMENT-IDENTIFIER: US 6689398 B2
TITLE: Triterpene compositions and methods for use thereof

Brief Summary Text (5):
Plants are valuable sources for the identification of novel biologically active molecules. One diverse class of molecules which has been identified in plants is the class of saponins. Saponins are high molecular weight compounds comprising glycosides with a sugar moiety linked to a triterpene or steroid aglycone. Triterpene saponins particularly have been the subject of much interest because of their biological properties.

Brief Summary Text (6):
Pharmacological and biological properties of triterpene saponins from different plant species have been studied, including fungicidal, anti-viral, anti-mutagenic, spermicidal or contraceptive, cardiovascular, and anti-inflammatory activities (Hostettmann et al., 1995). Saponins are known to form complexes with cholesterol by binding plasma lipids, thereby altering cholesterol metabolism (Oakenfull et al., 1983). Triterpene glycosides given in feed also have been shown to decrease the amount of cholesterol in the blood and tissues of experimental animals (Cheeke, 1971). Saponins have been found to be constituents of many folk medicine remedies and some of the more recently developed plant drugs.

Brief Summary Text (8):
Betulinic acid, a pentacyclic triterpene, is reported to be a selective inhibitor of human melanoma tumor growth in nude mouse xenograft models and was shown to cause cytotoxicity by inducing apoptosis (Pisha et al., 1995). A triterpene saponin from a Chinese medicinal plant in the Cucurbitaceae family has demonstrated anti-tumor activity (Kong et al., 1993). Monoglycosides of triterpenes have been shown to exhibit potent and selective cytotoxicity against MOLT-4 human leukemia cells (Kasiwada et al., 1992) and certain triterpene glycosides of the Iridaceae family inhibited the growth of tumors and increased the life span of mice implanted with Ehrlich ascites carcinoma (Nagamoto et al., 1988). A saponin preparation from the plant Dolichos falcatus, which belongs to the Leguminosae family, has been reported to be effective against sarcoma-37 cells in vitro and in vivo (Huang et al., 1982). Soya saponin, also from the Leguminosae family, has been shown to be effective against a number of tumors (Tomas-Barbaren et al., 1988). Oleanolic acid and gypsogenin glycosides exhibiting haemolytic and molluscicidal activity have been isolated from the ground fruit pods of Swartzia madagascariensis (Leguminosae) (Borel and Hostettmann, 1987).

Brief Summary Text (15):
An important aspect of the invention provides the isolation of a mixture comprising one or more isolated saponins or triterpene glycosides that may be characterized by the following properties: a) isolatable from the tissues of Acacia victoriae; b) containing at least one triterpene glycoside having a molecular weight of from about 1800 to about 2600 amu; c) the ability to induce cytotoxicity in a Jurkat cell; and d) the ability to induce apoptosis in a Jurkat cell.

Brief Summary Text (25):
In another aspect, the invention provides a process for preparing a composition comprising a mixture of one or more isolated triterpene glycosides, comprising: a)

obtaining tissue from an Acacia victoriae plant; b) extracting the tissue with a solvent to provide an extract; and c) obtaining one or more triterpene glycosides from the extract. The tissues used in this process typically comprises pods, roots, seedlings, or mixtures thereof. The solvent used for the extraction may be any organic solvent which is capable of extracting, often by dissolving, the saponin compound of interest. Useful extraction solvents are methanol, ethanol, isopropyl alcohol, dichloromethane, chloroform, ethyl acetate, water, glycerol and mixtures thereof.

Detailed Description Text (8):
An important aspect in the use of plant extracts as pharmaceutical preparations is the characterization and determination of the individual active constituents. Such also is the case for triterpene saponin preparations, which often require sophisticated techniques for the isolation, structure elucidation and analysis of their components and glycosides. When biological testing of the pure compounds is to be performed, it is necessary to isolate them in sufficient quantity and purity.

Detailed Description Text (9):
Since triterpenes and other related saponins have relatively large molecular weights and are of high polarity, their isolation can be challenging. A problem involved in the isolation of pure saponins is the presence of complex mixtures of closely related compounds, differing subtly either in the nature of the aglycone or the sugar part (nature, number, positions and chirality of attachment of the monosaccharides). Difficulties also are encountered with labile substituents such as esters. For example, the major genuine soybean saponin, a .gamma.-pyrone derivative (BOA), is only extracted by aqueous ethanol at room temperature. Extraction with heating (80.degree. C.) leads to fission of the ester moiety and formation of soyasaponin I (Bb) (Kudou et al., 1992). In plants, saponins are accompanied by very polar substances, such as saccharides and coloring matter, including phenolic compounds and the like, are not easily crystallized, and can be hygroscopic, making it even more difficult to obtain crystals.

Detailed Description Text (16):
(ii) Extraction and Preliminary Purification

Detailed Description Text (17):
Extraction procedures should be as mild as possible because certain saponins can undergo transformations including enzymatic hydrolysis during water extraction, esterification of acidic saponins during alcohol treatment, hydrolysis of labile ester groups and transacylation. Therefore, care should be taken to follow the individual steps in an isolation procedure, for example, in thin layer chromatography.

Detailed Description Text (18):
Although numerous variations are possible, current general procedures for obtaining crude saponin mixtures typically include extraction with methanol, ethanol, water or aqueous alcohol; a defatting step, generally with petroleum ether, performed before the extraction step or on the extract itself; dissolution or suspension of the extract in water; shaking or washing the solution or suspension with n-butanol saturated with water; and precipitation (optional) of saponins with diethyl ether or acetone. A dialysis step also can be included in order to remove small water-soluble molecules such as sugars (see, for example, Zhou et al., 1981; Massiot et al., 1988).

Detailed Description Text (19):
The most efficient extraction of dry plant material is achieved with methanol or aqueous methanol. Methanol is also used for fresh plant material. Although water is typically a less efficient extraction solvent for saponins (unless specifically water-soluble glycosides are desired) it has the advantages of being easily

lyophilized and giving a cleaner extract. Depending on the proportion of water used for extraction, either monodesmosidic or bidesmosidic saponins may be obtained (Domon and Hostettmann, 1984; Kawamura et al., 1988). Fresh vegetable material contains active enzymes (esterases) which, when homogenized with a solvent, are able to convert bidesmosides into mono-desmosides. Even dry material may contain esterases which are activated in the presence of water. In the case of momordin I (a monodesmosidic oleanolic acid saponin) it was found that conversion to momordin II (the corresponding bidesmoside) takes place in water and in 30% and 60% methanol solutions, but not in 80% and 100% methanol solutions. On the contrary, homogenates of the fresh roots in methanol retained enzyme activity. However, the enzymes could be inactivated by first soaking the fresh roots in 4% hydrochloric acid and the bidesmoside was then shown to be the major component. It is, therefore, clear that the correct choice of extraction procedure is an extremely important first step.

Detailed Description Text (21):
A common problem observed for furostanol saponins is the formation of 22-OCH.sub.3 derivatives during extraction with methanol. However, the genuine 22-hydroxyfurostanols can either be obtained by extraction with another solvent (e.g., pyridine) or by treatment of the methoxylated artifacts with boiling aqueous acetone (Konishi and Shoji, 1979).

Detailed Description Text (23):
The qualitative analysis of triterpene saponins by TLC is of great importance for all aspects of saponin investigations. TLC plates (usually silica gel) can handle both pure saponins and crude extracts, are inexpensive, rapid to use and require no specialized equipment. A number of visualization reagents are available for spraying onto the plates (Table 2). Methods of preparation of the most common reagents are as follows: Vanillin-sulfuric acid (Godin reagent). A 1% solution of vanillin in ethanol is mixed in a 1:1 ratio with a 3% solution of perchloric acid in water and sprayed onto the TLC plate. This is followed by a 10% solution of sulfuric acid in ethanol and heating at 110.degree. C. Liebermann-Burchard reagent. Concentrated sulfuric acid (1 ml) is mixed with acetic anhydride (20 ml) and chloroform (50 ml). Heating at 85-90.degree. C. gives the required coloration on the TLC plate. Antimony(III) chloride. The TLC plate is sprayed with a 10% solution of antimony chloride in chloroform and heated to 100.degree. C. Anisaldehyde-sulfuric acid. Anisaldehyde (0.5 ml) is mixed with glacial acetic acid (10 ml), methanol (85 ml) and concentrated sulfuric acid (5 ml). This solution is sprayed onto the TLC plate, which is then heated at 100.degree. C.

Detailed Description Text (24):
Spraying with vanillin-sulfuric acid in the presence of ethanol and perchloric acid, for example, gives a blue or violet coloration with triterpene saponins. With anisaldehyde-sulfuric acid, a blue or violet-blue coloration is produced on heating the TLC plate. Spraying TLC plates with a solution of cerium sulphate in sulfuric acid gives violet-red, blue or green fluorescent zones under 365 nm UV light (Kitagawa et al., 1984b). In some cases, simply spraying the plates with water is sufficient to reveal the saponins present. Additional spray reagents may be found in, for example, Stahl (1969).

Detailed Description Text (78):
A variety of separation techniques have been described and may be used for separating triterpene saponins including flash chromatography, DCCC, low-pressure liquid chromatography (LPLC), medium-pressure liquid chromatography (MPLC), HPLC and conventional open-column chromatography (See, e.g., Hostettmann et al., 1986, 1991; Marston and Hostettmann, 1991 b). An idea of separation conditions, solvent systems, etc. will be known to those of skill in the art in light of the instant disclosure. The best results are usually achieved by strategies which employ a combination of methods, such as those specifically disclosed herein below.

Detailed Description Text (101):

For example, a combination of MPLC on silica gel and RP material, LPLC and centrifugal TLC for separation of saponins (Hamburger and Hostettmann, 1986). Similarly, the isolation of five triterpene saponins from Swartzia madagascariensis (Leguminosae) required open-column chromatography, LPLC and MPLC (Borel and Hostettmann, 1987).

Detailed Description Text (104):
Another strategy involves passing extracts (after preliminary partition) over highly porous polymers and following this step by further fractionation of the crude saponin mixtures. This approach was used in the isolation of 3.beta.-hydroxyolean-12-en-28,29-dioic acid glycosides from Nothopanax delavayi (Araliaceae). A methanol extract of the leaves and stems was partitioned between hexane and water. The aqueous layer was chromatographed on a Diaion HP-20 column and eluted with water, 10% methanol, 50% methanol, 80% methanol, methanol and chloroform. The glycosides were obtained by subsequent column chromatography of the 80% methanol eluate on silica gel with ethyl acetate-ethanol-water (7:2:1) (Kasai et al., 1987a). For the isolation of triterpene and non-triterpene saponins from Acanthopanax senticosus (Araliaceae), the procedure began with a fractionation of the methanol extract of the leaves on Diaion HP-20 polymer. The fraction eluted with methanol was chromatographed on silica gel (chloroform-methanol-water 30:10:1) and all the resulting fractions were subjected to column chromatography on LiChroprep RP-8. Final purification was achieved by HPLC on TSK-GEL ODS-120T (300.times.21 min; methanol-water 70:30; 6 ml/min; RI detection) or chromatography on a hydroxyapatite column (acetonitrile-water 85:15) (Shao et al., 1988).

Detailed Description Text (107):
Reactions of triterpenes with any of a variety of agents may be used to produce colored compounds for the quantitative or qualitative determination of triterpenes. For example, aromatic aldehydes such as aisaldehyde and vanillin in strong mineral acid, for example, sulfuric, phosphoric, and perchloric acids, give colored products with aglycones, having absorption maxima between 510 and 620 nm. In these reactions, a dehydration is believed to occur, forming unsaturated methylene groups which give colored condensation products with the aldehydes. With vanillin-sulfuric acid, triterpene saponins with a C-23 hydroxyl group have a peak located between 460 and 485 nm (Hiai et al., 1976).

Detailed Description Text (112):
Legume extracts were prepared by chloroform:methanol or dichloromethane: chloroform extraction at The University of Arizona (Tucson, Ariz.). The inventors isolated mixtures of triterpene glycosides from Acacia victoriae (Benth.) (Leguminosae). The first collection of UA-BRF-004-DELEP-F001 was processed as follows: (1) grinding to 3 mm particle size in Wiley mill, (2) packing into two-liter percolation unit, (3) extracting the ground biomass with dichloromethane:methanol (1:1) for 4 hr. followed by overnight and the combined fractions were dried in vacuo to generate UA-BRF-004-DELEP-F001 (52 g). F001 (51.5 g) was extracted with ethyl acetate to yield active insoluble (34.7 g) material designated as F004. Flash chromatography using 1.7 kg of silica gel (Merck, 23-220 micron particle size) was used to fractionate F004 (34.2), 51 670-ml fractions eluted with dichloromethane: methanol (step-gradient-95--0%: methanol 5--100%). the Column was washed with nine-liters of methanol followed by six-liters of methanol:water (80:20) and then six-liters of same eluent with 1% formic acid added. Based on TLC fractions 23-34 and 39-40 were combined to 17.2 g of F023. Medium Pressure Liquid Chromatography (MPLC, Buchi 632 system) was used twice with 8 g of F023 each on a 4.9.times.46-cm column packed with Lichroprep C18, 15-25 micron particle size using step gradient of acetonitrile:water (0, 10, 20, 30, 50% acetonitrile in water) followed by 100% methanol wash. Of the 16 g 0-20% acetonitrile, yield was seven grams of F027, which was inactive. The remaining material was combined and subjected to repetitive MPLC with the same system using 30-40% acetonitrile to minimize overlap and generate fractions F028-F036. Although most of these fractions demonstrated antitumor activity, F035 (Fraction 35) (highest yield of 2.19 g) was selected for further

testing and evaluation.

Detailed Description Text (114):
Various methods may be employed for the qualitative and quantitative determination of triterpenes and their activities including: piscicidal activity, gravimetry, spectrophotometry, TLC, GC, HPLC, HMQC, HMBC, NOESY, COSY, NMR, X-Ray crystallography etc. Determinations based on classical properties of triterpene saponins (surface activity, fish toxicity) have largely been replaced by photometric methods such as densitometry, colorimetry of derivatives and, more recently, by GC, HPLC and particularly, NMR. Spectrophotometric methods are very sensitive but not typically suitable for estimating triterpenes in crude plant extracts since the reactions are not specific and colored products may form with compounds which accompany the triterpenes, such as phytosterols and flavonoids. Another problem, common to much of the analytical work on saponins, is their incomplete extraction from the plant material. However, a number of techniques are widely available which are suitable for quantitating triterpenes.

Detailed Description Text (122):
For assigning chemical shifts, it is helpful to compare observed data with data reported for model and related compounds. As a guide to some of the typical chemical shifts in the .sup.13 C-NMR spectrum of a triterpene saponin, one may use the known shifts of the bayogenin glycoside (Domon and Hostettmann, 1984). Additionally, compilations of assignments of .sup.13 C-NMR signals for oleanane (Patra et al, 1981; Agrawal and Jain, 1992), ursane, lupane (Wenkert et al, 1978; Sholichin et al., 1980), hopane (Wenkert et al., 1978; Wilkins et al., 1987) and lanostane (Parrilli et al., 1979) triterpenes have been made (Nakanishi et al., 1983). The relevant data for dammarane glycosides have been summarized in a review (Tanaka and Kasai, 1984), while .sup.13 C-NMR spectroscopy of saikogenins (Tori et al., 1976a) and of saikosaponins (Tori et al., 1976b) has been described. Ginseng sapogenins and related dammarane triterpenes also have been studied (Asakawa et al., 1977). .sup.13 C-NMR spectroscopy of acacic acid has also been described (Kinjo et al, 1992).

Detailed Description Text (131):
In practice, certain .sup.1 H and .sup.13 C NMR spectra can be identified and assigned on the basis of shift arguments, but for interpreting the results of NMR studies in a rigorous manner, an NMR spectrum should be assigned unambiguously, which means establishing which peaks are associated with which carbon and/or hydrogen in the structure. This information, in most cases, cannot be obtained from one-dimensional .sup.1 H and .sup.13 C NMR spectral data, but can better be determined with the aid of two-dimensional studies. These studies simplify spectral analysis by spreading out information into two frequency domains and by revealing interactions between nuclei. Despite the fact that the mechanisms on which the various pulse sequences are established may be intricate, the interpretation of two-dimensional NMR spectra is usually straightforward. A large number of different two-dimensional NMR studies have been devised to solve chemical structures. Examples of such techniques, as well as other NMR techniques specifically contemplated by the inventors for use in the chemical elucidation of the triterpene saponins of the invention, are described below, and in Table 3.

Detailed Description Text (141):
The sequences of sugar and interglycosidic linkages of triterpene glycosides from marine organisms have been established from NT.sub.1 data and NOESY studies (Miyamoto et al., 1990) but this methodology is limited by the complexity of the .sup.1 H-NMR spectra in the 3-5 p.p.m. region, which usually precludes the measurement of NOE for a large number of protons. However, a combination of COSY, NOESY and direct and XHCORR NMR spectroscopy has allowed complete signal assignment and structural analysis of pentasaccharide triterpene saponins from the sea cucumber Holothuria forskalii (Rodriguez et al., 1991).

Detailed Description Text (185):
Triterpene saponins are glycosides in which the hemiacetal hydroxyl groups of saccharides in their cyclic pyranose or furanose forms build acetals with a triterpene or steroid residue. The ether linkage between the hemiacetal hydroxyl and the triterpene or steroid is known as a glycosidic linkage. The monosaccharide constituents of the oligosaccharides also are bound by ether linkages (interglycosidic bonds).

Detailed Description Text (187):
Numerous chemical reactions and methods have been employed for breaking down saponins into smaller units for more ready analysis (see, for example, Kitagawa, 1981). Such methods will find particular use in structural determinations of triterpene saponins.

Detailed Description Text (189):
Acidic hydrolysis maybe carried out by refluxing the saponin in acid for a fixed length of time, for example, 4 h in 2-4 M hydrochloric acid. The aqueous solution remaining after hydrolysis is extracted with diethyl ether, chloroform or ethyl acetate to obtain the aglycone. Extraction of the sugars from the aqueous layer is performed with pyridine, after neutralizing the solution (with alkali or basic ion exchange resin) (Tschesche and Forstmann, 1957; Sandberg and Michel, 1962) and evaporation to dryness. The saponins are completely cleaved into their constituents by this method so information is obtained as to the identity of the aglycone and the number and nature of monosaccharides present. If a prosapogenin (obtained after cleavage of an ester linkage by basic hydrolysis) is acid hydrolyzed, the nature of the sugar chains which are ether-linked to the aglycone can be established. An aqueous reaction medium can be replaced by alcohol or dioxane.

Detailed Description Text (409):
Sixty plant species were chosen from the Desert Legume Project (DELEP) with the goal of identifying novel compounds having beneficial biological activities. The DELEP (University of Arizona, Tucson) is a collection of desert legume species developed through a collaboration between the University of Arizona and the Boyce Thompson Southwestern Arboretum. Experimental field samples were collected from each of the plant species, air-dried for 3-4 days, ground to three millimeter particle size with a Wiley mill (3 mm screen size) and extracted two or three times by percolation with a 1:1 mixture of dichloromethane (DCM) and methanol (MeOH). Each percolation extraction proceeded for at least 5 hours and often continued overnight. The majority of the extracted biomass was collected from the first two percolations. The biomass was then washed with a volume of methanol equal to half the void volume, and the crude extract contained in the methanol aliquots isolated. The samples were typically isolated and prepared for bioassay by removing the methanol in vacuo, passing the aqueous phase through RP-C18 particles, recovering the active constituents in MeOH, and then rotovapping the MeOH to collect the extract as a solid. The crude extract was then resuspended in H20, DMSO or mixtures thereof (less polar compounds were resuspended in DMSO, while more polar compounds were resuspended in water or water and DMSO mixtures; aglycones were resuspended in DMSO).

Detailed Description Text (427):
Although the above procedures focused on the isolation of active constituents from pods of Acacia victoriae, the active constituents may also be extracted from roots. In this case, the roots are ground for 1/2 hour and covered with 100% MeOH. The mixture is then filtered and diluted to 80% MeOH in water. If large amounts of roots are to be extracted, then it may be preferable to extract via percolation as described above. The reason for the differences in these extraction procedures is that roots are typically extracted fresh while the pods are often dried prior to extraction.

Detailed Description Text (430):

A modified extraction/separation procedure was used for the scaled-up preparation of mixtures of active constituents from fraction UA-BRF-004Pod-DELEP-F094 (F094). This procedure was repeated multiple times, consistently yielding highly active fractions. Typically, 20-25 g of F094 or its equivalent was dissolved in 150-175 ml of 50% MeOH in H.sub.2 O which was then aspirated onto a column ((26 mm.times.460 mm)+(70 mm.times.460 mm), RP-C18, 40 .mu.m, 1200 g, equilibrated with 60% MeOH/H.sub.2 O). The fractions were eluted in steps of 8 L in 60% MeOH/H.sub.2 O; 7.5 L 70% MeOH/H.sub.2 O; and 2 L MeOH and assigned fraction identifiers as shown in Table 15. Fraction F035-B2 contains a mixture of the active components contained in F094, F133-136 (isolated from F093) and F138-147 (isolated from F094) as shown in FIGS. 18A-18F. F094 is an acceptable substitute for F035 with a one- to two-fold decrease in potency and F035-B2 has less potency than F094.

Detailed Description Text (432):
Further improvements to the above extraction procedure, as well as the other extraction procedures disclosed herein, may be realized by using tri-solvent mixtures of acetonitrile, methanol and water. The percentage ranges can be dynamically produced and optimized by anyone familiar with standard chromatographic techniques. Likewise, bonded phase silicas can be varied by using a combination of RP systems, including, but not limited to C-8, CN, dimethyl diol and C-18. In the final steps, even normal phase silica can be utilized for final purification procedures.

Detailed Description Text (516):
The isolation of B1 was accomplished by plant extraction and C-18 flash chromatography followed by C-18 prep and semi-prep chromatography. The NMR of B1 indicates the same triterpene/monoterpene/quinovose/monoterpene structure as has been seen throughout this saponin family. The NMR also indicates the presence of four deoxy sugars and one N-acetyl group, which indicates that this molecule must differ from D1 in its sugar portions. See Table 21 for specific .sup.13 C-NMR assignments under (21). This molecule was degraded as shown in FIG. 38.

Detailed Description Text (548):
Next, in order to further study the mechanism by which the active components inhibited tumor cells, the TNF-alpha induced activation of the transcription factor NF-.kappa.B was analyzed in Jurkat cells (3.times.10.sup.6) which had been treated with 1-2 .mu.g/ml of UA-BRF-004-DELEP-F035 and UA-BRF-004Pod-DELEP-F094. The study was carried out as follows: Jurkat cells were pretreated with 1-2 .mu.g/ml of F035 or F094 for 15 h at 37.degree. C. Cells were harvested and resuspended in 1 ml RPMI and treated with 100 pM of TNF-alpha for 30 min at 37.degree. C. After TNF-alpha treatment, nuclear extracts were prepared according to Schreiber et al. (1989). Briefly, the cells were washed with ice cold PBS and suspended in 0.4 ml of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, 2 .mu.g/ml of leupeptin, 2 .mu.g/ml of aprotinin and 0.5 mg/ml benzamidine). The cells were allowed to sit on ice for 15 min and 25 .mu.l of 10% Nonidet-40 was added to the cells. The tubes were mixed on the vortex and microcentrifuged for 30 s. The nuclear pellet was resuspended in 25 .mu.l of ice cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 2 .mu.g/ml leupeptin, 2.0 .mu.g/ml aprotinin and 0.5 mg/ml benzamidine) and tubes were incubated on ice with intermittent agitation. The nuclear extract was microcentrifuged for 5 runs at 4.degree. C. and supernatants were stored at -70.degree. C.

Detailed Description Text (668):
Caspase-3 activity was measured as described earlier (Enari et al, 1995) with some modifications. Briefly, Jurkat cells (1.times.10.sup.6 /ml) were treated with F035, D1 & G1 for different lengths of time. Cytosolic extracts were prepared by repeated freeze thawing in 300 .mu.l of extraction buffer (12.5 mM Tris, pH 7.0, 1 mM DTT, 0.125 mM EDTA, 5% glycerol, 1 .mu.M PMSF, 1 .mu.g/ml leupeptin, 1 .mu.g/ml pepstatin and 1 .mu.g/ml aprotinin). Cell lystates were diluted 1:2 with ICE buffer

(50 mM Tris, pH 7.0, 0.5 mM EDTA, 4 mM DTT and 20% glycerol) and incubated with 20 .mu.M of a caspase 3 substrate (acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin) at 37.degree. C. Caspase-3 activity was monitored by the production of fluorescent aminomethylcoumarin, which was measured at excitation 355 nM, emission 460 nM using Fluoroscan II (Labsystems, Helsinki, Finland).

Detailed Description Text (692):
For harvesting of roots, the root mass of a single plant is rinsed with water directly in the aeroponic box and the root mass is cut with scissors a few inches above the sprayer. The excess water is removed by patting dry with paper towels, followed by weighing of the sample. The root mass is then cut in 3-4 inch sections with scissors and subject to chemical extraction, as described above. Alternatively, for continual harvest of roots, the pump is turned off and roots are clipped from the growing root mass. These roots are then cut into 3-4 inch sections and extracted as described. Care is taken not to damage the non-harvested roots.

Detailed Description Text (722):
Different media were tested for growth of hairy roots. Best growth was obtained on MS medium containing 2% sucrose. The effect of different capacity flasks and gibberellic acid was tested on the growth of hairy roots. The hairy roots were also grown on MS liquid medium on gyratory shaker in a 125 ml conical flask with 20 ml medium. An increase in growth of 84 fold was noted in 4 weeks. The production of triterpene saponins corresponding to those identified in F035 was confirmed by HPLC analysis with a standard authentic sample.

Detailed Description Paragraph Table (1):
TABLE 1 Applications of MPLC in the Separation of Triterpene Saponins Plant Support Solvent Reference Cussonia spicata Silica gel CHCl.sub.3 --MeOH--H.sub.2 0 Gunzinger et al., 1986 (6:4:1) C-8 MeOH--H.sub.2 0 (2:1) Gunzinger et al., 1986 Calendula arvensis C-8 MeOH--H.sub.2 0 (65:35, Chemli et al., 1987 73:27) C. officinalis Silica gel CHCl.sub.3 MeOH H.sub.2 0 Vidal-Ollivier et al., (61:32:5) 1989 C-18 MeOH--H.sub.2 0 (60:40, Vidal-Ollivier et al., 80:20) 1989 Polygala Silica gel CH.sub.2 Cl.sub.2 --MeOH H.sub.2 0 Hamburger and chamaebuxus (80:20:2) Hostettmann, 1986 C-8 MeOH--H.sub.2 0 (55:45) Hamburger and Hostettmann, 1986 Swartzia C-8 MeOH H.sub.2 0 (65:35) Borel and Hostettmann, madagascariensis 1987 Talinum C-8 MeOH--H.sub.2 0 (60:40) Gafner et al., 1985 tenuissimum Sesbania sesban C-8 MeOH--H.sub.2 0 (55:45, Dorsaz et al., 1988 60:40) Tetrapleura C-8 MeOH--H.sub.2 0 (70:30) Maillard et al., 1989 tetraptera Albizzia lucida C-8 MeOH--H.sub.2 0 (6:4 .fwdarw. 9:1) Orsini et al., 1991 C-18 MeOH--H.sub.2 0 (7:3) Orsini et al., 1991 Passiflora C-18 MeOH--H.sub.2 0 (17:3) Orsini and Verotta, quadrangularis 1985 Hedera helix C-18 MeOH--H.sub.2 0 gradient Elias et al., 1991 Primula veris C-18 MeOH--H.sub.2 0 (5:5 .fwdarw. 7:3) Calis et al., 1992 Silica gel CHCl.sub.3 --MeOH--H.sub.2 0 (61:32:7) Calis et al., 1992 Steroid saponins Balanites Silica gel CHCl.sub.3 --MeOH--H.sub.2 0 Hosny et al., 1992 aegyptiaca (80:20:1 .fwdarw. 25:25:2 and 70:30:3)

Detailed Description Paragraph Table (2):
TABLE 2 Visualization Reagents for Triterpene Saponins Reagent Reference Vanillin-sulfuric acid Godin, 1954 Vanillin-phosphoric acid Oakenfull, 1981 Liebermann-Burchard (acetic Abisch and Reichstein, 1960 anhydride-sulfuric acid) Wagner et al., 1984 1% Cerium sulphate in 10% sulfuric acid Kitagawa et al., 1984b 10% Sulfuric acid in ethanol Price et al., 1987 50% Sulfuric acid Price et al., 1987 p-Anisaldehyde-sulfuric acid Wagner et al., 1984 Komarowsky Wagner et al., 1985 (p-hydroxybenzaldehyde-sulfuric acid) Antimony(III) chloride Wagner et al., 1984 Blood Wagner et al., 1984 Water

Detailed Description Paragraph Table (3):
TABLE 3 Selected NMR Approaches for Use in the Structure Establishment of Triterpene Saponins NMR Study (Acronyms) Comments Attached proton test (APT), Distortionless Discriminates among carbon types; enhancement by polarization

transfer (DEPT), Spectral editing Insensitive nuclei enhancement by polarization transfer (INEPT) Incredible natural abundance double-quantum .sup.13 C--.sup.13 C connectivity, establishment transfer study (INADEQUATE) of molecular skeleton .sup.1 H, .sup.1 H-COSY Homonuclear shift correlation a) normal Elucidation of direct couplings b) with delays Detection of small couplings c) double-quantum filtered-(DQF) - COSY Determination of vicinal and geminal coupling constants d) Exclusive COSY (E. COSY) Accurate determination of J e) Geminal COSY (Gem - COSY) Identification of geminal spin systems f) Triple-quantum filtered (TQF) - COSY Detection of three or more mutually coupled spin systems Relayed coherence transfer (RCT), Total correlation Identification of all protons (TOCSY), and Hartmann-Hahn study (HOHAHA) belonging to a single spin system; Coherence transfer across scalar connectivity (particularly useful in identifying monosaccharide residues) Homonuclear nuclear Overhauser and exchange Identification of protons that are spectroscopy (NOESY and ROESY) within 5A of one another (.sup.1 H, .sup.1 H correlation through space); Stereochemical analysis (orientation of substituents); Intra- and inter-residual connectivities (sequence analysis in sugar chain including sugar - aglycone linkage) 1H{.sup.13c C}SBC (HETCOR and HMQC) Heteronuclear shift correlation; Assignments of directly bonded .sup.1 H and .sup.13 C shifts HMQC-TOCSY and HMQC-RELAY Cross assignments of .sup.1 H and .sup.13 C shifts 1H{.sup.13 C}MBC (Long-range HETCOR and HMBC) Assignment of quaternary C; Correlation of a proton resonance with a carbon resonance 2-4 bonds distant; Intra- and inter-residual assignments (inter-glycosidic and sugar-aglycone linkage); Confirmation of molecular structure

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**** See image for Certificate of Correction ****
TITLE: Triterpene compositions and methods for use thereof

Brief Summary Text (5):
Plants are valuable sources for the identification of novel biologically active molecules. One diverse class of molecules which has been identified in plants is the class of saponins. Saponins are high molecular weight compounds comprising glycosides with a sugar moiety linked to a triterpene or steroid aglycone. Triterpene saponins particularly have been the subject of much interest because of their biological properties.

Brief Summary Text (6):
Pharmacological and biological properties of triterpene saponins from different plant species have been studied, including fungicidal, anti-viral, anti-mutagenic, spermicidal or contraceptive, cardiovascular, and anti-inflammatory activities (Hostettmann et al, 1995). Saponins are known to form complexes with cholesterol by binding plasma lipids, thereby altering cholesterol metabolism (Oakenfull et al., 1983). Triterpene glycosides given in feed also have been shown to decrease the amount of cholesterol in the blood and tissues of experimental animals (Cheeke, 1971). Saponins have been found to be constituents of many folk medicine remedies and some of the more recently developed plant drugs.

Brief Summary Text (8):
Betulinic acid, a pentacyclic triterpene, is reported to be a selective inhibitor of human melanoma tumor growth in nude mouse xenograft models and was shown to cause cytotoxicity by inducing apoptosis (Pisha et al., 1995). A triterpene saponin from a Chinese medicinal plant in the Cucurbitaceae family has demonstrated anti-tumor activity (Kong et al., 1993). Monoglycosides of triterpenes have been shown to exhibit potent and selective cytotoxicity against MOLT-4 human leukemia cells (Kasiwada et al., 1992) and certain triterpene glycosides of the Iridaceae family inhibited the growth of tumors and increased the life span of mice implanted with Ehrlich ascites carcinoma (Nagamoto et al., 1988). A saponin preparation from the plant Dolichos falcatus, which belongs to the Leguminosae family, has been reported to be effective against sarcoma-37 cells in vitro and in vivo (Huang et al., 1982). Soya saponin, also from the Leguminosae family, has been shown to be effective against a number of tumors (Tomas-Barbaren et al., 1988). Oleanolic acid and gypsogenin glycosides exhibiting haemolytic and molluscicidal activity have been isolated from the ground fruit pods of Swartzia madagascariensis (Leguminosae) (Borel and Hostettmann, 1987).

Brief Summary Text (15):
An important aspect of the invention provides the isolation of a mixture comprising one or more isolated saponins or triterpene glycosides that may be characterized by the following properties: a) isolatable from the tissues of Acacia victoriae; b) containing at least one triterpene glycoside having a molecular weight of from about 1800 to about 2600 amu; c) the ability to induce cytotoxicity in a Jurkat cell; and d) the ability to induce apoptosis in a Jurkat cell.

Brief Summary Text (25):
In another aspect, the invention provides a process for preparing a composition comprising a mixture of one or more isolated triterpene glycosides, comprising: a) obtaining tissue from an Acacia victoriae plant; b) extracting the tissue with a solvent to provide an extract; and c) obtaining one or more triterpene glycosides from the extract. The tissues used in this process typically comprises pods, roots, seedlings, or mixtures thereof. The solvent used for the extraction may be any organic solvent which is capable of extracting, often by dissolving, the saponin compound of interest. Useful extraction solvents are methanol, ethanol, isopropyl alcohol, dichloromethane, chloroform, ethyl acetate, water, glycerol and mixtures thereof.

Detailed Description Text (8):
An important aspect in the use of plant extracts as pharmaceutical preparations is the characterization and determination of the individual active constituents. Such also is the case for triterpene saponin preparations, which often require sophisticated techniques for the isolation, structure elucidation and analysis of their components and glycosides. When biological testing of the pure compounds is to be performed, it is necessary to isolate them in sufficient quantity and purity.

Detailed Description Text (9):
Since triterpenes and other related saponins have relatively large molecular weights and are of high polarity, their isolation can be challenging. A problem involved in the isolation of pure saponins is the presence of complex mixtures of closely related compounds, differing subtly either in the nature of the aglycone or the sugar part (nature, number, positions and chirality of attachment of the monosaccharides). Difficulties also are encountered with labile substituents such as esters. For example, the major genuine soybean saponin, a .gamma.-pyrone derivative (BOA), is only extracted by aqueous ethanol at room temperature. Extraction with heating (80.degree. C.) leads to fission of the ester moiety and formation of soyasaponin I (Bb) (Kudou et al., 1992). In plants, saponins are accompanied by very polar substances, such as saccharides and coloring matter, including phenolic compounds and the like, are not easily crystallized, and can be

hygroscopic, making it even more difficult to obtain crystals.

Detailed Description Text (16):
(ii) Extraction and Preliminary Purification

Detailed Description Text (17):
Extraction procedures should be as mild as possible because certain saponins can undergo transformations including enzymatic hydrolysis during water extraction, esterification of acidic saponins during alcohol treatment, hydrolysis of labile ester groups and transacylation. Therefore, care should be taken to follow the individual steps in an isolation procedure, for example, in thin layer chromatography.

Detailed Description Text (18):
Although numerous variations are possible, current general procedures for obtaining crude saponin mixtures typically include extraction with methanol, ethanol, water or aqueous alcohol; a defatting step, generally with petroleum ether, performed before the extraction step or on the extract itself; dissolution or suspension of the extract in water; shaking or washing the solution or suspension with n-butanol saturated with water; and precipitation (optional) of saponins with diethyl ether or acetone. A dialysis step also can be included in order to remove small water-soluble molecules such as sugars (see, for example, Zhou et al., 1981; Massiot et al., 1988).

Detailed Description Text (19):
The most efficient extraction of dry plant material is achieved with methanol or aqueous methanol. Methanol is also used for fresh plant material. Although water is typically a less efficient extraction solvent for saponins (unless specifically water-soluble glycosides are desired) it has the advantages of being easily lyophilized and giving a cleaner extract. Depending on the proportion of water used for extraction, either monodesmosidic or bidesmosidic saponins may be obtained (Domon and Hostettmann, 1984; Kawamura et al., 1988). Fresh vegetable material contains active enzymes (esterases) which, when homogenized with a solvent, are able to convert bidesmosides into mono-desmosides. Even dry material may contain esterases which are activated in the presence of water. In the case of momordin I (a monodesmosidic oleanolic acid saponin) it was found that conversion to momordin II (the corresponding bidesmoside) takes place in water and in 30% and 60% methanol solutions, but not in 80% and 100% methanol solutions. On the contrary, homogenates of the fresh roots in methanol retained enzyme activity. However, the enzymes could be inactivated by first soaking the fresh roots in 4% hydrochloric acid and the bidesmoside was then shown to be the major component. It is, therefore, clear that the correct choice of extraction procedure is an extremely important first step.

Detailed Description Text (21):
A common problem observed for furostanol saponins is the formation of 22-OCH.sub.3 derivatives during extraction with methanol. However, the genuine 22-hydroxyfurostanols can either be obtained by extraction with another solvent (e.g., pyridine) or by treatment of the methoxylated artifacts with boiling aqueous acetone (Konishi and Shoji, 1979).

Detailed Description Text (23):
The qualitative analysis of triterpene saponins by TLC is of great importance for all aspects of saponin investigations. TLC plates (usually silica gel) can handle both pure saponins and crude extracts, are inexpensive, rapid to use and require no specialized equipment. A number of visualization reagents are available for spraying onto the plates (Table 2). Methods of preparation of the most common reagents are as follows:

Detailed Description Text (28):
Spraying with vanillin-sulfuric acid in the presence of ethanol and perchloric

acid, for example, gives a blue or violet coloration with triterpene saponins. With anisaldehyde-sulfiric acid, a blue or violet-blue coloration is produced on heating the TLC plate. Spraying TLC plates with a solution of cerium sulphate in sulfuric acid gives violet-red, blue or green fluorescent zones under 365 nm UV light (Kitagawa et al., 1984b). In some cases, simply spraying the plates with water is sufficient to reveal the saponins present Additional spray reagents may be found in, for example, Stahl (1969).

Detailed Description Text (82):
A variety of separation techniques have been described and may be used for separating triterpene saponins including flash chromatography, DCCC, low-pressure liquid chromatography (LPLC), medium-pressure liquid chromatography (MPLC), HPLC and conventional open-column chromatography (See, e.g., Hostettmann et al., 1986, 1991; Marston and Hostettmann, 1991 b). An idea of separation conditions, solvent systems, etc. will be known to those of skill in the art in light of the instant disclosure. The best results are usually achieved by strategies which employ a combination of methods, such as those specifically disclosed herein below.

Detailed Description Text (105):
For example, a combination of MPLC on silica gel and RP material, LPLC and centrifugal TLC for separation of saponins (Hamburger and Hostettmann, 1986). Similarly, the isolation of five triterpene saponins from Swartzia madagascariensis (Leguminosae) required open-column chromatography, LPLC and MPLC (Borel and Hostettmann, 1987).

Detailed Description Text (108):
Another strategy involves passing extracts (after preliminary partition) over highly porous polymers and following this step by further fractionation of the crude saponin mixtures. This approach was used in the isolation of 3.beta.-hydroxyolean-12-en-28,29-dioic acid glycosides from Nothopanax delavayi (Araliaceae). A methanol extract of the leaves and stems was partitioned between hexane and water. The aqueous layer was chromatographed on a Diaion HP-20 column and eluted with water, 10% methanol, 50% methanol, 80% methanol, methanol and chloroform. The glycosides were obtained by subsequent column chromatography of the 80% methanol eluate on silica gel with ethyl acetate-ethanol-water (7:2:1) (Kasai et al., 1987a). For the isolation of triterpene and non-triterpene saponins from Acanthopanax senticosus (Araliaceae), the procedure began with a fractionation of the methanol extract of the leaves on Diaion HP-20 polymer. The fraction eluted with methanol was chromatographed on silica gel (chloroform-methanol-water 30:10:1) and all the resulting fractions were subjected to column chromatography on LiChroprep RP-8. Final purification was achieved by HPLC on TSK-GEL ODS-120T (300.times.21 min; methanol-water 70:30; 6 ml/min; RI detection) or chromatography on a hydroxyapatite column (acetonitrile-water 85:15) (Shao et al., 1988).

Detailed Description Text (111):
Reactions of triterpenes with any of a variety of agents may be used to produce colored compounds for the quantitative or qualitative determination of triterpenes. For example, aromatic aldehydes such as aisaldehyde and vanillin in strong mineral acid, for example, sulfuric, phosphoric, and perchloric acids, give colored products with aglycones, having absorption maxima between 510 and 620 nm. In these reactions, a dehydration is believed to occur, forming unsaturated methylene groups which give colored condensation products with the aldehydes. With vanillin-sulfiric acid, triterpene saponins with a C-23 hydroxyl group have a peak located between 460 and 485 nm (Hiai et al., 1976).

Detailed Description Text (116):
Legume extracts were prepared by chloroform:methanol or dichloromethane: chloroform extraction at The University of Arizona (Tucson, Ariz.). The inventors isolated mixtures of triterpene glycosides from Acacia victoriae (Benth.) (Leguminosae). The first collection of UA-BRF-004-DELEP-F001 was processed as follows: (1) grinding to

3 mm particle size in Wiley mill, (2) packing into two-liter percolation unit, (3) extracting the ground biomass with dichloromethane:methanol (1:1) for 4 hr. followed by overnight and the combined fractions were dried in vacuo to generate UA-BRF-004-DELEP-F001 (52 g). F001 (51.5 g) was extracted with ethyl acetate to yield active insoluble (34.7 g) material designated as F004. Flash chromatography using 1.7 kg of silica gel (Merck, 23-220 micron particle size) was used to fractionate F004 (34.2), 51 670-ml fractions eluted with dichloromethane: methanol (step-gradient-95-0%: methanol 5-100%). the Column was washed with nine-liters of methanol followed by six-liters of methanol:water (80:20) and then six-liters of same eluent with 1% formic acid added. Based on TLC fractions 23-34 and 39-40 were combined to 17.2 g of F023. Medium Pressure Liquid Chromatography (MPLC, Buchi 632 system) was used twice with 8 g of F023 each on a 4.9.times.46-cm column packed with Lichroprep C18, 15-25 micron particle size using step gradient of acetonitrile: water (0,10,20,30,50% acetonitrile in water) followed by 100% methanol wash. Of the 16 g 0-20% acetonitrile, yield was seven grams of F027, which was inactive. The remaining material was combined and subjected to repetitive MPLC with the same system using 30-40% acetonitrile to minimize overlap and generate fractions F028-F036. Although most of these fractions demonstrated antitumor activity, F035 (Fraction 35) (highest yield of 2.19 g) was selected for further testing and evaluation.

Detailed Description Text (118):
Various methods may be employed for the qualitative and quantitative determination of triterpenes and their activities including: piscicidal activity, gravimetry, spectrophotometry, TLC, GC, HPLC, HMQC, HMBC, NOESY, COSY, NMR, X-Ray crystallography etc. Determinations based on classical properties of triterpene saponins (surface activity, fish toxicity) have largely been replaced by photometric methods such as densitometry, colorimetry of derivatives and, more recently, by GC, HPLC and particularly, NMR. Spectrophotometric methods are very sensitive but not typically suitable for estimating triterpenes in crude plant extracts since the reactions are not specific and colored products may form with compounds which accompany the triterpenes, such as phytosterols and flavonoids. Another problem, common to much of the analytical work on saponins, is their incomplete extraction from the plant material. However, a number of techniques are widely available which are suitable for quantitating triterpenes.

Detailed Description Text (126):
For assigning chemical shifts, it is helpful to compare observed data with data reported for model and related compounds. As a guide to some of the typical chemical shifts in the .sup.13 C-NMR spectrum of a triterpene saponin, one may use the known shifts of the bayogenin glycoside (Domon and Hostettmann, 1984). Additionally, compilations of assignments of .sup.13 C-NMR signals for oleanane (Patra et al., 1981; Agrawal and Jain, 1992), ursane, lupane (Wenkert et al., 1978; Sholichin et al., 1980), hopane (Wenkert et al, 1978; Wilkins et al., 1987) and lanostane (Parrilli et al., 1979) triterpenes have been made (Nakanishi et al., 1983). The relevant data for dammarane glycosides have been summarized in a review (Tanaka and Kasai, 1984), while .sup.13 C-NMR spectroscopy of saikogenins (Tori et al., 1976a) and of saikosaponins (Tori et al, 1976b) has been described. Ginseng sapogenins and related dammarane triterpenes also have been studied (Asakawa et al., 1977). .sup.13 C-NMR spectroscopy of acacic acid has also been described (Kinjo et al., 1992).

Detailed Description Text (135):
In practice, certain .sup.1 H and .sup.13 C NMR spectra can be identified and assigned on the basis of shift arguments, but for interpreting the results of NMR studies in a rigorous manner, an NMR spectrum should be assigned unambiguously, which means establishing which peaks are associated with which carbon and/or hydrogen in the structure. This information, in most cases, cannot be obtained from one-dimensional .sup.1 H and .sup.13 C NMR spectral data, but can better be determined with the aid of two-dimensional studies. These studies simplify spectral

analysis by spreading out information into two frequency domains and by revealing interactions between nuclei. Despite the fact that the mechanisms on which the various pulse sequences are established may be intricate, the interpretation of two-dimensional NMR spectra is usually straightforward. A large number of different two-dimensional NMR studies have been devised to solve chemical structures. Examples of such techniques, as well as other NMR techniques specifically contemplated by the inventors for use in the chemical elucidation of the triterpene saponins of the invention, are described below, and in Table 3.

Detailed Description Text (145):
The sequences of sugar and interglycosidic linkages of triterpene glycosides from marine organisms have been established from NT.sub.1 data and NOESY studies (Miyamoto et al., 1990) but this methodology is limited by the complexity of the .sup.1 H-NMR spectra in the 3-5 p.p.m. region, which usually precludes the measurement of NOE for a large number of protons. However, a combination of COSY, NOESY and direct and XHCORR NMR spectroscopy has allowed complete signal assignment and structural analysis of pentasaccharide triterpene saponins from the sea cucumber Holothuria forskalii (Rodriguez et al., 1991).

Detailed Description Text (189):
Triterpene saponins are glycosides in which the hemiacetal hydroxyl groups of saccharides in their cyclic pyranose or furanose forms build acetals with a triterpene or steroid residue. The ether linkage between the hemiacetal hydroxyl and the triterpene or steroid is known as a glycosidic linkage. The monosaccharide constituents of the oligosaccharides also are bound by ether linkages (interglycosidic bonds).

Detailed Description Text (191):
Numerous chemical reactions and methods have been employed for breaking down saponins into smaller units for more ready analysis (see, for example, Kitagawa, 1981). Such methods will find particular use in structural determinations of triterpene saponins.

Detailed Description Text (193):
Acidic hydrolysis maybe carried out by refluxing the saponin in acid for a fixed length of time, for example, 4 h in 2-4 M hydrochloric acid. The aqueous solution remaining after hydrolysis is extracted with diethyl ether, chloroform or ethyl acetate to obtain the aglycone. Extraction of the sugars from the aqueous layer is performed with pyridine, after neutralizing the solution (with alkali or basic ion exchange resin) (Tschesche and Forstmann, 1957; Sandberg and Michel, 1962) and evaporation to dryness. The saponins are completely cleaved into their constituents by this method so information is obtained as to the identity of the aglycone and the number and nature of monosaccharides present. If a prosapogenin (obtained after cleavage of an ester linkage by basic hydrolysis) is acid hydrolyzed, the nature of the sugar chains which are ether-linked to the aglycone can be established. An aqueous reaction medium can be replaced by alcohol or dioxane.

Detailed Description Text (413):
Sixty plant species were chosen from the Desert Legume Project (DELEP) with the goal of identifying novel compounds having beneficial biological activities. The DELEP (University of Arizona, Tucson) is a collection of desert legume species developed through a collaboration between the University of Arizona and the Boyce Thompson Southwestern Arboretum. Experimental field samples were collected from each of the plant species, air-dried for 3-4 days, ground to three millimeter particle size with a Wiley mill (3 mm screen size) and extracted two or three times by percolation with a 1:1 mixture of dichloromethane (DCM) and methanol (MeOH). Each percolation extraction proceeded for at least 5 hours and often continued overnight. The majority of the extracted biomass was collected from the first two percolations. The biomass was then washed with a volume of methanol equal to half the void volume, and the crude extract contained in the methanol aliquots isolated.

The samples were typically isolated and prepared for bioassay by removing the methanol in vacuo, passing the aqueous phase through RP-C18 particles, recovering the active constituents in MeOH, and then rotovapping the MeOH to collect the extract as a solid. The crude extract was then resuspended in H20, DMSO or mixtures thereof (less polar compounds were resuspended in DMSO, while more polar compounds were resuspended in water or water and DMSO mixtures; aglycones were resuspended in DMSO).

Detailed Description Text (431):
Although the above procedures focused on the isolation of active constituents from pods of Acacia victoriae, the active constituents may also be extracted from roots. In this case, the roots are ground for 1/2 hour and covered with 100% MeOH. The mixture is then filtered and diluted to 80% MeOH in water. If large amounts of roots are to be extracted, then it may be preferable to extract via percolation as described above. The reason for the differences in these extraction procedures is that roots are typically extracted fresh while the pods are often dried prior to extraction.

Detailed Description Text (434):
A modified extraction/separation procedure was used for the scaled-up preparation of mixtures of active constituents from fraction UA-BRF-004Pod-DELEP-F094 (F094). This procedure was repeated multiple times, consistently yielding highly active fractions. Typically, 20-25 g of F094 or its equivalent was dissolved in 150-175 ml of 50% MeOH in H.sub.2 O which was then aspirated onto a column ((26 mm.times.460 mm)+(70 mm.times.460 mm), RP-C 18, 40 .mu.m, 1200 g, equilibrated with 60% MeOH/H.sub.2 O). The fractions were eluted in steps of 8 L in 60% MeOH/H.sub.2 O; 7.5 L 70% MeOH.sub.2 O; and 2 L MeOH and assigned fraction identifiers as shown in Table 15. Fraction F035-B2 contains a mixture of the active components contained in F094, F133-136 (isolated from F093) and F138-147 (isolated from F094) as shown in FIGS. 18A-18F. F094 is an acceptable substitute for F035 with a one- to two-fold decrease in potency and F035-B2 has less potency than F094.

Detailed Description Text (436):
Further improvements to the above extraction procedure, as well as the other extraction procedures disclosed herein, may be realized by using tri-solvent mixtures of acetonitrile, methanol and water. The percentage ranges can be dynamically produced and optimized by anyone familiar with standard chromatographic techniques. Likewise, bonded phase silicas can be varied by using a combination of RP systems, including, but not limited to C-8, CN, dimethyl diol and C-18. In the final steps, even normal phase silica can be utilized for final purification procedures.

Detailed Description Text (518):
The isolation of B1 was accomplished by plant extraction and C-18 flash chromatography followed by C-18 prep and semi-prep chromatography. The NMR of B1 indicates the same triterpene/monoterpene/quinovose/monoterpene structure as has been seen throughout this saponin family. The NMR also indicates the presence of four deoxy sugars and one N-acetyl group, which indicates that this molecule must differ from D1 in its sugar portions. See Table 21 for specific .sup.13 C-NMR assignments under (21). This molecule was degraded as shown in FIG. 38.

Detailed Description Text (550):
Next, in order to further study the mechanism by which the active components inhibited tumor cells, the TNF-alpha induced activation of the transcription factor NF-.kappa.B was analyzed in Jurkat cells (3.times.10.sup.6) which had been treated with 1-2 .mu.g/ml of UA-BRF-004-DELEP-F035 and UA-BRF-004Pod-DELEP-F094. The study was carried out as follows: Jurkat cells were pretreated with 1-2 .mu.g/ml of F035 or F094 for 15 h at 37.degree. C. Cells were harvested and resuspended in 1 ml RPMI and treated with 100 pM of TNF-alpha for 30 min at 37.degree. C. After TNF-alpha treatment, nuclear extracts were prepared according to Schreiber et al. (1989).

Briefly, the cells were washed with ice cold PBS and suspended in 0.4 ml of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, 2 .mu.g/ml of leupeptin, 2 .mu.g/ml of aprotinin and 0.5 mg/ml benzamidine). The cells were allowed to sit on ice for 15 min and 25 .mu.l of 10% Nonidet-40 was added to the cells. The tubes were mixed on the vortex and microcentrifuged for 30 s. The nuclear pellet was resuspended in 25 .mu.l of ice cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 2 .mu.g/ml leupeptin, 2.0 .mu.g/ml aprotinin and 0.5 mg/ml benzamidine) and tubes were incubated on ice with intermittent agitation. The nuclear extract was microcentrifuged for 5 runs at 4.degree. C. and supernatants were stored at -70.degree. C.

Detailed Description Text (672):
Caspase-3 activity was measured as described earlier (Enari et al., 1995) with some modifications. Briefly, Jurkat cells (1.times.10.sup.6/ ml) were treated with F035, D1 & G1 for different lengths of time. Cytosolic extracts were prepared by repeated freeze thawing in 300 .mu.l of extraction buffer (12.5 mM Tris, pH 7.0, 1 mM DTT, 0.125 mM EDTA, 5% glycerol, 1 .mu.M PMSF, 1 .mu.g/ml leupeptin, 1 .mu.g/ml pepstatin and 1 .mu.g/ml aprotinin). Cell lystates were diluted 1:2 with ICE buffer (50 mM Tris, pH 7.0, 0.5 mM EDTA, 4 mM DTT and 20% glycerol) and incubated with 20 .mu.M of a caspase 3 substrate (acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin) at 37.degree. C. Caspase-3 activity was monitored by the production of fluorescent aminomethylcoumarin, which was measured at excitation 355 nM, emission 460 nM using Fluoroscan II (Labsystems, Helsinki, Finland).

Detailed Description Text (696):
For harvesting of roots, the root mass of a single plant is rinsed with water directly in the aeroponic box and the root mass is cut with scissors a few inches above the sprayer. The excess water is removed by patting dry with paper towels, followed by weighing of the sample. The root mass is then cut in 3-4 inch sections with scissors and subject to chemical extraction, as described above. Alternatively, for continual harvest of roots, the pump is turned off and roots are clipped from the growing root mass. These roots are then cut into 3-4 inch sections and extracted as described. Care is taken not to damage the non-harvested roots.

Detailed Description Text (726):
Different media were tested for growth of hairy roots. Best growth was obtained on MS medium containing 2% sucrose. The effect of different capacity flasks and gibberellic acid was tested on the growth of hairy roots. The hairy roots were also grown on MS liquid medium on gyratory shaker in a 125 ml conical flask with 20 ml medium. An increase in growth of 84 fold was noted in 4 weeks. The production of triterpene saponins corresponding to those identified in F035 was confirmed by HPLC analysis with a standard authentic sample.

Detailed Description Paragraph Table (1):
TABLE 1 Applications of MPLC in the Separation of Triterpene Saponins Plant Support Solvent Reference Cussonia spicata Silica CHCl.sub.3 --MeOH--H.sub.2 0 Gunzinger gel (6:4:1) et al., 1986 C-8 MeOH--H.sub.2 0 (2:1) Gunzinger et al., 1986 Calendula C-8 MeOH--H.sub.2 0 (65:35, Chemli arvensis 73:27) et al., 1987 C. officinalis Silica CHCl.sub.3 MeOH H.sub.2 0 Vidal- gel (61:32:5) Ollivier et al., 1989 C-18 MeOH--H.sub.2 0 (60:40, Vidal- 80:20) Ollivier et al., 1989 Polygala Silica CH.sub.2 Cl.sub.2 --MeOH H.sub.2 0 Hamburger chamaebuxus gel (80:20:2) and Hostettmann, 1986 C-8 MeOH--H.sub.2 0 (55:45) Hamburger and Hostettmann, 1986 Swartzia C-8 MeOH H.sub.2 0 (65:35) Borel and madagascariensis Hostettmann, 1987 Talinum C-8 MeOH--H.sub.2 0 (60:40) Gafner tenuissimum et al., 1985 Sesbania sesban C-8 MeOH--H.sub.2 0 (55:45, Dorsaz 60:40) et al., 1988 Tetrapleura C-8 MeOH--H.sub.2 0 (70:30) Maillard tetraptera et al., 1989 Albizzia lucida C-8 MeOH--H.sub.2 0 (6:4 .fwdarw. 9:1) Orsini et al., 1991 C-18 MeOH--H.sub.2 0 (7:3) Orsini et al., 1991 Passiflora C-18 MeOH--H.sub.2 0 (17:3) Orsini and quadrangularis Verotta, 1985 Hedera helix C-18 MeOH--H.sub.2 0 gradient Elias et al., 1991 Primula

veris C-18 MeOH--H.sub.2 0 (5:5 .fwdarw. 7:3) Calis et al., 1992 Silica CHCl.sub.3 --MeOH--H.sub.2 0 (61:3 Calis gel 2:7) et al., 1992 Steroid saponins Balanites Silica CHCl.sub.3 --MeOH--H.sub.2 0 Hosny aegyptiaca gel (80:20:1 .fwdarw. 25:25:2 and et al., 1992 70:30:3)

Detailed Description Paragraph Table (2):
TABLE 2 Visualization Reagents for Triterpene Saponins Reagent Reference Vanillin-sulfuric acid Godin, 1954 Vanillin-phosphoric acid Oakenfull, 1981 Liebermann-Burchard (acetic Abisch and Reichstein, 1960 anhydride-sulfuric acid) Wagner et al., 1984 1% Cerium sulphate in 10% sulfuric acid Kitagawa et al., 1984b 10% Sulfuric acid in ethanol Price et al., 1987 50% Sulfuric acid Price et al., 1987 p-Anisaldehyde-sulfuric acid Wagner et al., 1984 Komarowsky Wagner et al., 1985 (p-hydroxybenzaldehyde-sulfuric acid) Antimony (III) chloride Wagner et al., 1984 Blood Wagner et al., 1984 Water

Detailed Description Paragraph Table (3):
TABLE 3 Selected NMR Approaches for Use in the Structure Establishment of Triterpene Saponins NMR Study (Acronyms) Comments Attached proton test (APT), Discriminates among carbon types; Distortionless enhancement by Spectral editing polarization transfer (DEPT), Insensitive nuclei enhancement by polarization transfer (INEPT) Incredible natural abundance .sup.13 C--.sup.13 C connectivity, establishment double-quantum transfer study of molecular skeleton (INADEQUATE) .sup.1 H, .sup.1 H-COSY Homonuclear shift correlation a) normal Elucidation of direct couplings b) with delays Detection of small couplings c) double-quantum Determination of vicinal and filtered-(DQF)-COSY geminal coupling constants d) Exclusive COSY (E. COSY) Accurate determination of J e) Geminal COSY (Gem-COSY) Identification of geminal spin systems f) Triple-quantum filtered Detection of three or more (TQF)-COSY mutually coupled spin systems Relayed coherence transfer Identification of all protons (RCT), Total correlation belonging to a single spin system; (TOCSY), and Hartmann-Hahn study (HOHAHA) Coherence transfer across scalar connectivity (particularly useful in identifying monosaccharide residues) Homonuclear nuclear Overhauser Identification of protons that are and exchange spectroscopy within 5A of one another (.sup.1 H, .sup.1 H (NOESY and ROESY) correlation through space); Stereochemical analysis (orientation of substituents); Intra- and inter-residual connectivities (sequence analysis in sugar chain including sugar-aglycone linkage) 1H{.sup.13c C}SBC (HETCOR and HMQC) Heteronuclear shift correlation; Assignments of directly bonded .sup.1 H and .sup.13 C shifts HMQC-TOCSY and HMQC-RELAY Cross assignments of .sup.1 H and .sup.13 C shifts 1H{.sup.13 C}MBC (Long-range Assignment of quaternary C; HETCOR and HMBC) Correlation of a proton resonance with a carbon resonance 2-4 bonds distant; Intra- and inter-residual assignments (inter-glycosidic and sugar-aglycone linkage); Confirmation of molecular structure

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**** See image for Certificate of Correction ****
TITLE: Triterpene compositions and methods for use thereof

Brief Summary Text (5):
Plants are valuable sources for the identification of novel biologically active molecules. One diverse class of molecules which has been identified in plants is the class of saponins. Saponins are high molecular weight compounds comprising glycosides with a sugar moiety linked to a triterpene or steroid aglycone. Triterpene saponins particularly have been the subject of much interest because of their biological properties.

Brief Summary Text (6):
Pharmacological and biological properties of triterpene saponins from different plant species have been studied, including fungicidal, anti-viral, anti-mutagenic, spermicidal or contraceptive, cardiovascular, and anti-inflammatory activities (Hostettmann et al, 1995). Saponins are known to form complexes with cholesterol by binding plasma lipids, thereby altering cholesterol metabolism (Oakenfull et al., 1983). Triterpene glycosides given in feed also have been shown to decrease the amount of cholesterol in the blood and tissues of experimental animals (Cheeke, 1971). Saponins have been found to be constituents of many folk medicine remedies and some of the more recently developed plant drugs.

Brief Summary Text (8):
Betulinic acid, a pentacyclic triterpene, is reported to be a selective inhibitor of human melanoma tumor growth in nude mouse xenograft models and was shown to cause cytotoxicity by inducing apoptosis (Pisha et al., 1995). A triterpene saponin from a Chinese medicinal plant in the Cucurbitaceae family has demonstrated anti-tumor activity (Kong et al., 1993). Monoglycosides of triterpenes have been shown to exhibit potent and selective cytotoxicity against MOLT-4 human leukemia cells (Kasiwada et al., 1992) and certain triterpene glycosides of the Iridaceae family inhibited the growth of tumors and increased the life span of mice implanted with Ehrlich ascites carcinoma (Nagamoto et al., 1988). A saponin preparation from the plant Dolichos falcatus, which belongs to the Leguminosae family, has been reported to be effective against sarcoma-37 cells in vitro and in vivo (Huang et al., 1982). Soya saponin, also from the Leguminosae family, has been shown to be effective against a number of tumors (Tomas-Barbaren et al., 1988). Oleanolic acid and gypsogenin glycosides exhibiting haemolytic and molluscicidal activity have been isolated from the ground fruit pods of Swartzia madagascariensis (Leguminosae) (Borel and Hostettmann, 1987).

Brief Summary Text (15):
An important aspect of the invention provides the isolation of a mixture comprising one or more isolated saponins or triterpene glycosides that may be characterized by the following properties: a) isolatable from the tissues of Acacia victoriae; b) containing at least one triterpene glycoside having a molecular weight of from about 1800 to about 2600 amu; c) the ability to induce cytotoxicity in a Jurkat cell; and d) the ability to induce apoptosis in a Jurkat cell.

Brief Summary Text (25):
In another aspect, the invention provides a process for preparing a composition comprising a mixture of one or more isolated triterpene glycosides, comprising: a) obtaining tissue from an Acacia victoriae plant; b) extracting the tissue with a solvent to provide an extract; and c) obtaining one or more triterpene glycosides from the extract. The tissues used in this process typically comprises pods, roots, seedlings, or mixtures thereof. The solvent used for the extraction may be any organic solvent which is capable of extracting, often by dissolving, the saponin compound of interest. Useful extraction solvents are methanol, ethanol, isopropyl alcohol, dichloromethane, chloroform, ethyl acetate, water, glycerol and mixtures thereof.

Detailed Description Text (8):
An important aspect in the use of plant extracts as pharmaceutical preparations is the characterization and determination of the individual active constituents. Such

also is the case for triterpene saponin preparations, which often require sophisticated techniques for the isolation, structure elucidation and analysis of their components and glycosides. When biological testing of the pure compounds is to be performed, it is necessary to isolate them in sufficient quantity and purity.

Detailed Description Text (9):
Since triterpenes and other related saponins have relatively large molecular weights and are of high polarity, their isolation can be challenging. A problem involved in the isolation of pure saponins is the presence of complex mixtures of closely related compounds, differing subtly either in the nature of the aglycone or the sugar part (nature, number, positions and chirality of attachment of the monosaccharides). Difficulties also are encountered with labile substituents such as esters. For example, the major genuine soybean saponin, a .gamma.-pyrone derivative (BOA), is only extracted by aqueous ethanol at room temperature. Extraction with heating (80.degree. C.) leads to fission of the ester moiety and formation of soyasaponin I (Bb) (Kudou et al., 1992). In plants, saponins are accompanied by very polar substances, such as saccharides and coloring matter, including phenolic compounds and the like, are not easily crystallized, and can be hygroscopic, making it even more difficult to obtain crystals.

Detailed Description Text (16):
(ii) Extraction and Preliminary Purification

Detailed Description Text (17):
Extraction procedures should be as mild as possible because certain saponins can undergo transformations including enzymatic hydrolysis during water extraction, esterification of acidic saponins during alcohol treatment, hydrolysis of labile ester groups and transacylation. Therefore, care should be taken to follow the individual steps in an isolation procedure, for example, in thin layer chromatography.

Detailed Description Text (18):
Although numerous variations are possible, current general procedures for obtaining crude saponin mixtures typically include extraction with methanol, ethanol, water or aqueous alcohol; a defatting step, generally with petroleum ether, performed before the extraction step or on the extract itself; dissolution or suspension of the extract in water; shaking or washing the solution or suspension with n-butanol saturated with water; and precipitation (optional) of saponins with diethyl ether or acetone. A dialysis step also can be included in order to remove small water-soluble molecules such as sugars (see, for example, Zhou et al., 1981; Massiot et al., 1988).

Detailed Description Text (19):
The most efficient extraction of dry plant material is achieved with methanol or aqueous methanol. Methanol is also used for fresh plant material. Although water is typically a less efficient extraction solvent for saponins (unless specifically water-soluble glycosides are desired) it has the advantages of being easily lyophilized and giving a cleaner extract. Depending on the proportion of water used for extraction, either monodesmosidic or bidesmosidic saponins may be obtained (Domon and Hostettmann, 1984; Kawamura et al., 1988). Fresh vegetable material contains active enzymes (esterases) which, when homogenized with a solvent, are able to convert bidesmosides into mono-desmosides. Even dry material may contain esterases which are activated in the presence of water. In the case of momordin I (a monodesmosidic oleanolic acid saponin) it was found that conversion to momordin II (the corresponding bidesmoside) takes place in water and in 30% and 60% methanol solutions, but not in 80% and 100% methanol solutions. On the contrary, homogenates of the fresh roots in methanol retained enzyme activity. However, the enzymes could be inactivated by first soaking the fresh roots in 4% hydrochloric acid and the bidesmoside was then shown to be the major component. It is, therefore, clear that

the correct choice of extraction procedure is an extremely important first step.

Detailed Description Text (21):
A common problem observed for furostanol saponins is the formation of 22-OCH.sub.3 derivatives during extraction with methanol. However, the genuine 22-hydroxyfurostanols can either be obtained by extraction with another solvent (e.g., pyridine) or by treatment of the methoxylated artifacts with boiling aqueous acetone (Konishi and Shoji, 1979).

Detailed Description Text (23):
The qualitative analysis of triterpene saponins by TLC is of great importance for all aspects of saponin investigations. TLC plates (usually silica gel) can handle both pure saponins and crude extracts, are inexpensive, rapid to use and require no specialized equipment. A number of visualization reagents are available for spraying onto the plates (Table 2). Methods of preparation of the most common reagents are as follows:

Detailed Description Text (28):
Spraying with vanillin-sulfuric acid in the presence of ethanol and perchloric acid, for example, gives a blue or violet coloration with triterpene saponins. With anisaldehyde-sulfuric acid, a blue or violet-blue coloration is produced on heating the TLC plate. Spraying TLC plates with a solution of cerium sulphate in sulfuric acid gives violet-red, blue or green fluorescent zones under 365 nm UV light (Kitagawa et al., 1984b). In some cases, simply spraying the plates with water is sufficient to reveal the saponins present. Additional spray reagents may be found in, for example, Stahl (1969).

Detailed Description Text (82):
A variety of separation techniques have been described and may be used for separating triterpene saponins including flash chromatography, DCCC, low-pressure liquid chromatography (LPLC), medium-pressure liquid chromatography (MPLC), HPLC and conventional open-column chromatography (See, e.g., Hostettmann et al., 1986, 1991; Marston and Hostettmann, 1991 b). An idea of separation conditions, solvent systems, etc. will be known to those of skill in the art in light of the instant disclosure. The best results are usually achieved by strategies which employ a combination of methods, such as those specifically disclosed herein below.

Detailed Description Text (105):
For example, a combination of MPLC on silica gel and RP material, LPLC and centrifugal TLC for separation of saponins (Hamburger and Hostettmann, 1986). Similarly, the isolation of five triterpene saponins from Swartzia madagascariensis (Leguminosae) required open-column chromatography, LPLC and MPLC (Borel and Hostettmann, 1987).

Detailed Description Text (108):
Another strategy involves passing extracts (after preliminary partition) over highly porous polymers and following this step by further fractionation of the crude saponin mixtures. This approach was used in the isolation of 3.beta.-hydroxyolean-12-en-28,29-dioic acid glycosides from Nothopanax delavayi (Araliaceae). A methanol extract of the leaves and stems was partitioned between hexane and water. The aqueous layer was chromatographed on a Diaion HP-20 column and eluted with water, 10% methanol, 50% methanol, 80% methanol, methanol and chloroform. The glycosides were obtained by subsequent column chromatography of the 80% methanol eluate on silica gel with ethyl acetate-ethanol-water (7:2:1) (Kasai et al., 1987a). For the isolation of triterpene and non-triterpene saponins from Acanthopanax senticosus (Araliaceae), the procedure began with a fractionation of the methanol extract of the leaves on Diaion HP-20 polymer. The fraction eluted with methanol was chromatographed on silica gel (chloroform-methanol-water 30:10:1) and all the resulting fractions were subjected to column chromatography on LiChroprep RP-8. Final purification was achieved by HPLC on TSK-GEL ODS-120T

(300.times.21 min; methanol-water 70:30; 6 ml/min; RI detection) or chromatography on a hydroxyapatite column (acetonitrile-water 85:15) (Shao et al., 1988).

Detailed Description Text (111):
Reactions of triterpenes with any of a variety of agents may be used to produce colored compounds for the quantitative or qualitative determination of triterpenes. For example, aromatic aldehydes such as aisaldehyde and vanillin in strong mineral acid, for example, sulfuric, phosphoric, and perchloric acids, give colored products with aglycones, having absorption maxima between 510 and 620 nm. In these reactions, a dehydration is believed to occur, forming unsaturated methylene groups which give colored condensation products with the aldehydes. With vanillin-sulfuric acid, triterpene saponins with a C-23 hydroxyl group have a peak located between 460 and 485 nm (Hiai et al., 1976).

Detailed Description Text (116):
Legume extracts were prepared by chloroform:methanol or dichloromethane: chloroform extraction at The University of Arizona (Tucson, Ariz.). The inventors isolated mixtures of triterpene glycosides from Acacia victoriae (Benth.) (Leguminosae). The first collection of UA-BRF-004-DELEP-F001 was processed as follows: (1) grinding to 3 mm particle size in Wiley mill, (2) packing into two-liter percolation unit, (3) extracting the ground biomass with dichloromethane:methanol (1:1) for 4 hr. followed by overnight and the combined fractions were dried in vacuo to generate UA-BRF-004-DELEP-F001 (52 g). F001 (51.5 g) was extracted with ethyl acetate to yield active insoluble (34.7 g) material designated as F004. Flash chromatography using 1.7 kg of silica gel (Merck, 23-220 micron particle size) was used to fractionate F004 (34.2), 51 670-ml fractions eluted with dichloromethane: methanol (step-gradient-95-0%: methanol 5-100%). the Column was washed with nine-liters of methanol followed by six-liters of methanol:water (80:20) and then six-liters of same eluent with 1% formic acid added. Based on TLC fractions 23-34 and 39-40 were combined to 17.2 g of F023. Medium Pressure Liquid Chromatography (MPLC, Buchi 632 system) was used twice with 8 g of F023 each on a 4.9.times.46-cm column packed with Lichroprep C18, 15-25 micron particle size using step gradient of acetonitrile: water (0,10,20,30,50% acetonitrile in water) followed by 100% methanol wash. Of the 16 g 0-20% acetonitrile, yield was seven grams of F027, which was inactive. The remaining material was combined and subjected to repetitive MPLC with the same system using 30-40% acetonitrile to minimize overlap and generate fractions F028-F036. Although most of these fractions demonstrated antitumor activity, F035 (Fraction 35) (highest yield of 2.19 g) was selected for further testing and evaluation.

Detailed Description Text (118):
Various methods may be employed for the qualitative and quantitative determination of triterpenes and their activities including: piscicidal activity, gravimetry, spectrophotometry, TLC, GC, HPLC, HMQC, HMBC, NOESY, COSY, NMR, X-Ray crystallography etc. Determinations based on classical properties of triterpene saponins (surface activity, fish toxicity) have largely been replaced by photometric methods such as densitometry, colorimetry of derivatives and, more recently, by GC, HPLC and particularly, NMR. Spectrophotometric methods are very sensitive but not typically suitable for estimating triterpenes in crude plant extracts since the reactions are not specific and colored products may form with compounds which accompany the triterpenes, such as phytosterols and flavonoids. Another problem, common to much of the analytical work on saponins, is their incomplete extraction from the plant material. However, a number of techniques are widely available which are suitable for quantitating triterpenes.

Detailed Description Text (126):
For assigning chemical shifts, it is helpful to compare observed data with data reported for model and related compounds. As a guide to some of the typical chemical shifts in the .sup.13 C-NMR spectrum of a triterpene saponin, one may use the known shifts of the bayogenin glycoside (Domon and Hostettmann, 1984).

Additionally, compilations of assignments of .sup.13 C-NMR signals for oleanane (Patra et al., 1981; Agrawal and Jain, 1992), ursane, lupane (Wenkert et al, 1978; Sholichin et al., 1980), hopane (Wenkert et al., 1978; Wilkins et al., 1987) and lanostane (Parrilli et al, 1979) triterpenes have been made (Nakanishi et al., 1983). The relevant data for dammarane glycosides have been summarized in a review (Tanaka and Kasai, 1984), while .sup.13 C-NMR spectroscopy of saikogenins (Tori et al., 1976a) and of saikosaponins (Tori et al., 1976b) has been described. Ginseng sapogenins and related dammarane triterpenes also have been studied (Asakawa et al., 1977). .sup.13 C-NMR spectroscopy of acacic acid has also been described (Kinjo et al., 1992).

Detailed Description Text (135):
In practice, certain .sup.1 H and .sup.13 C NMR spectra can be identified and assigned on the basis of shift arguments, but for interpreting the results of NMR studies in a rigorous manner, an NMR spectrum should be assigned unambiguously, which means establishing which peaks are associated with which carbon and/or hydrogen in the structure. This information, in most cases, cannot be obtained from one-dimensional .sup.1 H and .sup.13 C NMR spectral data, but can better be determined with the aid of two-dimensional studies. These studies simplify spectral analysis by spreading out information into two frequency domains and by revealing interactions between nuclei. Despite the fact that the mechanisms on which the various pulse sequences are established may be intricate, the interpretation of two-dimensional NMR spectra is usually straightforward. A large number of different two-dimensional NMR studies have been devised to solve chemical structures. Examples of such techniques, as well as other NMR techniques specifically contemplated by the inventors for use in the chemical elucidation of the triterpene saponins of the invention, are described below, and in Table 3.

Detailed Description Text (145):
The sequences of sugar and interglycosidic linkages of triterpene glycosides from marine organisms have been established from NT.sub.1 data and NOESY studies (Miyamoto et al., 1990) but this methodology is limited by the complexity of the .sup.1 H-NMR spectra in the 3-5 p.p.m. region, which usually precludes the measurement of NOE for a large number of protons. However, a combination of COSY, NOESY and direct and XHCORR NMR spectroscopy has allowed complete signal assignment and structural analysis of pentasaccharide triterpene saponins from the sea cucumber Holothuria forskalii (Rodriguez et al., 1991).

Detailed Description Text (189):
Triterpene saponins are glycosides in which the hemiacetal hydroxyl groups of saccharides in their cyclic pyranose or furanose forms build acetals with a triterpene or steroid residue. The ether linkage between the hemiacetal hydroxyl and the triterpene or steroid is known as a glycosidic linkage. The monosaccharide constituents of the oligosaccharides also are bound by ether linkages (interglycosidic bonds).

Detailed Description Text (191):
Numerous chemical reactions and methods have been employed for breaking down saponins into smaller units for more ready analysis (see, for example, Kitagawa, 1981). Such methods will find particular use in structural determinations of triterpene saponins.

Detailed Description Text (193):
Acidic hydrolysis maybe carried out by refluxing the saponin in acid for a fixed length of time, for example, 4 h in 2-4 M hydrochloric acid. The aqueous solution remaining after hydrolysis is extracted with diethyl ether, chloroform or ethyl acetate to obtain the aglycone. Extraction of the sugars from the aqueous layer is performed with pyridine, after neutralizing the solution (with alkali or basic ion exchange resin) (Tschesche and Forstmann, 1957; Sandberg and Michel, 1962) and evaporation to dryness. The saponins are completely cleaved into their constituents

by this method so information is obtained as to the identity of the aglycone and the number and nature of monosaccharides present. If a prosapogenin (obtained after cleavage of an ester linkage by basic hydrolysis) is acid hydrolyzed, the nature of the sugar chains which are ether-linked to the aglycone can be established. An aqueous reaction medium can be replaced by alcohol or dioxane

Detailed Description Text (414):
Sixty plant species were chosen from the Desert Legume Project (DELEP) with the goal of identifying novel compounds having beneficial biological activities. The DELEP (University of Arizona, Tucson) is a collection of desert legume species developed through a collaboration between the University of Arizona and the Boyce Thompson Southwestern Arboretum. Experimental field samples were collected from each of the plant species, air-dried for 3-4 days, ground to three millimeter particle size with a Wiley mill (3 mm screen size) and extracted two or three times by percolation with a 1:1 mixture of dichloromethane (DCM) and methanol (MeOH). Each percolation extraction proceeded for at least 5 hours and often continued overnight. The majority of the extracted biomass was collected from the first two percolations. The biomass was then washed with a volume of methanol equal to half the void volume, and the crude extract contained in the methanol aliquots isolated. The samples were typically isolated and prepared for bioassay by removing the methanol in vacuo, passing the aqueous phase through RP-C18 particles, recovering the active constituents in MeOH, and then rotovapping the MeOH to collect the extract as a solid. The crude extract was then resuspended in H20, DMSO or mixtures thereof (less polar compounds were resuspended in DMSO, while more polar compounds were resuspended in water or water and DMSO mixtures; aglycones were resuspended in DMSO).

Detailed Description Text (432):
Although the above procedures focused on the isolation of active constituents from pods of Acacia victoriae, the active constituents may also be extracted from roots. In this case, the roots are ground for 1/2 hour and covered with 100% MeOH. The mixture is then filtered and diluted to 80% MeOH in water. If large amounts of roots are to be extracted, then it may be preferable to extract via percolation as described above. The reason for the differences in these extraction procedures is that roots are typically extracted fresh while the pods are often dried prior to extraction.

Detailed Description Text (435):
A modified extraction/separation procedure was used for the scaled-up preparation of mixtures of active constituents from fraction UA-BRF-004Pod-DELEP-F094 (F094). This procedure was repeated multiple times, consistently yielding highly active fractions. Typically, 20-25 g of F094 or its equivalent was dissolved in 150-175 ml of 50% MeOH in H.sub.2 O which was then aspirated onto a column ((26 mm.times.460 mm)+(70 mm.times.460 mm), RP-C 18, 40 .mu.m, 1200 g, equilibrated with 60% MeOH/H.sub.2 O). The fractions were eluted in steps of 8L in 60% MeOH/H.sub.2 O; 7.5 L 70% MeOH/H.sub.2 O; and 2L MeOH and assigned fraction identifiers as shown in Table 15. Fraction F035-B2 contains a mixture of the active components contained in F094, F133-136 (isolated from F093) and F138-147 (isolated from F094) as shown in FIGS. 18A-18F. F094 is an acceptable substitute for F035 with a one- to two-fold decrease in potency and F035-B2 has less potency than F094.

Detailed Description Text (437):
Further improvements to the above extraction procedure, as well as the other extraction procedures disclosed herein, may be realized by using tri-solvent mixtures of acetonitrile, methanol and water. The percentage ranges can be dynamically produced and optimized by anyone familiar with standard chromatographic techniques. Likewise, bonded phase silicas can be varied by using a combination of RP systems, including, but not limited to C-8, CN, dimethyl diol and C-18. In the final steps, even normal phase silica can be utilized for final purification procedures.

Detailed Description Text (520):
The isolation of B1 was accomplished by plant extraction and C-18 flash chromatography followed by C-18 prep and semi-prep chromatography. The NMR of B1 indicates the same triterpene/monoterpene/quinovose/monoterpene structure as has been seen throughout this saponin family. The NMR also indicates the presence of four deoxy sugars and one N-acetyl group, which indicates that this molecule must differ from D1 in its sugar portions. See Table 21 for specific .sup.13 C-NMR assignments under (21). This molecule was degraded as shown in FIG. 38.

Detailed Description Text (552):
Next, in order to further study the mechanism by which the active components inhibited tumor cells, the TNF-alpha induced activation of the transcription factor NF-.kappa.B was analyzed in Jurkat cells (3.times.10.sup.6) which had been treated with 1-2 .mu.g/ml of UA-BRF-004-DELEP-F035 and UA-BRF-004Pod-DELEP-F094. The study was carried out as follows: Jurkat cells were pretreated with 1-2 .mu.g/ml of F035 or F094 for 15 h at 37.degree. C. Cells were harvested and resuspended in 1 ml RPMI and treated with 100 pM of TNF-alpha for 30 min at 37.degree. C. After TNF-alpha treatment, nuclear extracts were prepared according to Schreiber et al. (1989). Briefly, the cells were washed with ice cold PBS and suspended in 0.4 ml of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, 2 .mu.g/ml of leupeptin, 2 .mu.g/ml of aprotinin and 0.5 mg/ml benzamidine). The cells were allowed to sit on ice for 15 min and 25 .mu.l of 10% Nonidet40 was added to the cells. The tubes were mixed on the vortex and microcentrifuged for 30 s. The nuclear pellet was resuspended in 25 .mu.l of ice cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 2 .mu.g/ml leupeptin, 2.0 .mu.g/ml aprotinin and 0.5 mg/ml benzamidine) and tubes were incubated on ice with intermittent agitation. The nuclear extract was microcentrifuged for 5 runs at 4.degree. C. and supernatants were stored at -70.degree. C.

Detailed Description Text (676):
Caspase-3 activity was measured as described earlier (Enari et al., 1995) with some modifications. Briefly, Jurkat cells (1.times.10.sup.6 ml) were treated with F035, D1 & G1 for different lengths of time. Cytosolic extracts were prepared by repeated freeze thawing in 300 .mu.l of extraction buffer (12.5 mM Tris, pH 7.0, 1 mM DTT, 0.125 mM EDTA, 5% glycerol, 1 .mu.M PMSF, 1 .mu.g/ml leupeptin, 1 .mu.g/ml pepstatin and 1 .mu.g/ml aprotinin). Cell lystates were diluted 1:2 with ICE buffer (50 mM Tris, pH 7.0, 0.5 mM EDTA, 4 mM DTT and 20% glycerol) and incubated with 20 .mu.M of a caspase 3 substrate (acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin) at 37.degree. C. Caspase-3 activity was monitored by the production of fluorescent aminomethylcoumarin, which was measured at excitation 355 nM, emission 460 nM using Fluoroscan II (Labsystems, Helsinki, Finland).

Detailed Description Text (700):
For harvesting of roots, the root mass of a single plant is rinsed with water directly in the aeroponic box and the root mass is cut with scissors a few inches above the sprayer. The excess water is removed by patting dry with paper towels, followed by weighing of the sample. The root mass is then cut in 3-4 inch sections with scissors and subject to chemical extraction, as described above. Alternatively, for continual harvest of roots, the pump is turned off and roots are clipped from the growing root mass. These roots are then cut into 3-4 inch sections and extracted as described. Care is taken not to damage the non-harvested roots.

Detailed Description Text (730):
Different media were tested for growth of hairy roots. Best growth was obtained on MS medium containing 2% sucrose. The effect of different capacity flasks and gibberellic acid was tested on the growth of hairy roots. The hairy roots were also grown on MS liquid medium on gyratory shaker in a 125 ml conical flask with 20 ml medium. An increase in growth of 84 fold was noted in 4 weeks. The production of

triterpene saponins corresponding to those identified in F035 was confirmed by HPLC analysis with a standard authentic sample.

Detailed Description Paragraph Table (1):
TABLE 1 Applications of MPLC in the Separation of Triterpene Saponins Plant Support Solvent Reference Cussonia spicata Silica gel CHCl.sub.3 --MeOH--H.sub.2 0 Gunzinger et al., 1986 (6:4:1) C-8 MeOH--H.sub.2 0 (2:1) Gunzinger et al., 1986 Calendula arvensis C-8 MeOH--H.sub.2 0 (65:35, Chemli et al., 1987 73:27) C. officinalis Silica gel CHCl.sub.3 MeOH H.sub.2 0 Vidal-Ollivier et al., (61:32:5) 1989 C-18 MeOH--H.sub.2 0 (60:40, Vidal-Ollivier et al., 80:20) 1989 Polygala Silica gel CH.sub.2 Cl.sub.2 --MeOH H.sub.2 0 Hamburger and chamaebuxus (80:20:2) Hostettmann, 1986 C-8 MeOH--H.sub.2 0 (55:45) Hamburger and Hostettmann, 1986 Swartzia C-8 MeOH H.sub.2 0 (65:35) Borel and Hostettmann, madagascariensis 1987 Talinum C-8 MeOH--H.sub.2 0 (60:40) Gafner et al., 1985 tenuissimum Sesbania sesban C-8 MeOH--H.sub.2 0 (55:45, Dorsaz et al., 1988 60:40) Tetrapleura C-8 MeOH--H.sub.2 0 (70:30) Maillard et al., 1989 tetraptera Albizzia lucida C-8 MeOH--H.sub.2 0 (6:4 .fwdarw. 9:1) Orsini et al., 1991 C-18 MeOH--H.sub.2 0 (7:3) Orsini et al., 1991 Passiflora C-18 MeOH--H.sub.2 0 (17:3) Orsini and Verotta, quadrangularis 1985 Hedera helix C-18 MeOH--H.sub.2 0 gradient Elias et al., 1991 Primula veris C-18 MeOH--H.sub.2 0 (5:5 .fwdarw. 7:3) Calis et al., 1992 Silica gel CHCl.sub.3 --MeOH--H.sub.2 0 Calis et al., 1992 (61:32:7) Steroid saponins Balanites Silica gel CHCl.sub.3 --MeOH--H.sub.2 0 Hosny et al., 1992 aegyptiaca (80:20:1 .fwdarw. 25:25:2 and 70:30:3)

Detailed Description Paragraph Table (2):
TABLE 2 Visualization Reagents for Triterpene Saponins Reagent Reference Vanillin-sulfuric acid Godin, 1954 Vanillin-phosphoric acid Oakenfull, 1981 Liebermann-Burchard (acetic Abisch and Reichstein, 1960 anhydride-sulfuric acid) Wagner et al., 1984 1% Cerium sulphate in Kitagawa et al., 1984b 10% sulfuric acid 10% Sulfuric acid in ethanol Price et al., 1987 50% Sulfuric acid Price et al., 1987 p-Anisaldehyde-sulfuric acid Wagner et al., 1984 Komarowsky Wagner et al., 1985 (p-hydroxybenzaldehyde- sulfuric acid) Antimony(III) chloride Wagner et al., 1984 Blood Wagner et al., 1984 Water

Detailed Description Paragraph Table (3):
TABLE 3 Selected NMR Approaches for Use in the Structure Establishment of Triterpene Saponins NMR Study (Acronyms) Comments Attached proton test (APT), Distortionless Discriminates among carbon types; enhancement by polarization transfer (DEPT), Spectral editing Insensitive nuclei enhancement by polarization transfer (INEPT) Incredible natural abundance double-quantum .sup.13 C--.sup.13 C connectivity, establishment transfer study (INADEQUATE) of molecular skeleton .sup.1 H, .sup.1 H-COSY Homonuclear shift correlation a) normal Elucidation of direct couplings b) with delays Detection of small couplings c) double-quantum filtered-(DQF) - COSY Determination of vicinal and geminal coupling constants d) Exclusive COSY (E. COSY) Accurate determination of J e) Geminal COSY (Gem - COSY) Identification of geminal spin systems f) Triple-quantum filtered (TQF) - COSY Detection of three or more mutually coupled spin systems Relayed coherence transfer (RCT), Total correlation Identification of all protons (TOCSY), and Hartmann-Hahn study (HOHAHA) belonging to a single spin system; Coherence transfer across scalar connectivity (particularly useful in identifying monosaccharide residues) Homonuclear nuclear Overhauser and exchange Identification of protons that are spectroscopy (NOESY and ROESY) within 5 A of one another (.sup.1 H, .sup.1 H correlation through space); Stereochemical analysis (orientation of substituents); Intra- and inter-residual connectivities (sequence analysis in sugar chain including sugar - aglycone linkage) 1H{.sup.13c C}SBC (HETCOR and HMQC) Heteronuclear shift correlation; Assignments of directly bonded .sup.1 H and .sup.13 C shifts HMQC-TOCSY and HMQC-RELAY Cross assignments of .sup.1 H and .sup.13 C shifts 1H{.sup.13 C}MBC (Long-range HETCOR and HMBC) Assignment of quaternary C; Correlation of a proton resonance with a carbon resonance 2-4 bonds distant; Intra- and inter-residual assignments (inter-

glycosidic and sugar-aglycone linkage); Confirmation of molecular structure

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L4: Entry 51 of 82 File: USPT Sep 3, 2002

DOCUMENT-IDENTIFIER: US 6444233 B1
TITLE: Triterpene compositions and methods for use thereof

Brief Summary Text (5):
Plants are valuable sources for the identification of novel biologically active molecules. One diverse class of molecules which has been identified in plants is the class of saponins. Saponins are high molecular weight compounds comprising glycosides with a sugar moiety linked to a triterpene or steroid aglycone. Triterpene saponins particularly have been the subject of much interest because of their biological properties.

Brief Summary Text (6):
Pharmacological and biological properties of triterpene saponins from different plant species have been studied, including fungicidal, anti-viral, anti-mutagenic, spermicidal or contraceptive, cardiovascular, and anti-inflammatory activities (Hostettmann et al, 1995). Saponins are known to form complexes with cholesterol by binding plasma lipids, thereby altering cholesterol metabolism (Oakenfull et al., 1983). Triterpene glycosides given in feed also have been shown to decrease the amount of cholesterol in the blood and tissues of experimental animals (Cheeke, 1971). Saponins have been found to be constituents of many folk medicine remedies and some of the more recently developed plant drugs.

Brief Summary Text (8):
Betulinic acid, a pentacyclic triterpene, is reported to be a selective inhibitor of human melanoma tumor growth in nude mouse xenograft models and was shown to cause cytotoxicity by inducing apoptosis (Pisha et al., 1995). A triterpene saponin from a Chinese medicinal plant in the Cucurbitaceae family has demonstrated anti-tumor activity (Kong et al., 1993). Monoglycosides of triterpenes have been shown to exhibit potent and selective cytotoxicity against MOLT-4 human leukemia cells (Kasiwada et al., 1992) and certain triterpene glycosides of the Iridaceae family inhibited the growth of tumors and increased the life span of mice implanted with Ehrlich ascites carcinoma (Nagamoto et al., 1988). A saponin preparation from the plant Dolichos falcatus, which belongs to the Leguminosae family, has been reported to be effective against sarcoma-37 cells in vitro and in vivo (Huang et al., 1982). Soya saponin, also from the Leguminosae family, has been shown to be effective against a number of tumors (Tomas-Barbaren et al., 1988). Oleanolic acid and gypsogenin glycosides exhibiting haemolytic and molluscicidal activity have been isolated from the ground fruit pods of Swartzia madagascariensis (Leguminosae) (Borel and Hostettmann, 1987).

Brief Summary Text (15):
An important aspect of the invention provides the isolation of a mixture comprising one or more isolated saponins or triterpene glycosides that may be characterized by the following properties: a) isolatable from the tissues of Acacia victoriae; b) containing at least one triterpene glycoside having a molecular weight of from

about 1800 to about 2600 amu; c) the ability to induce cytotoxicity in a Jurkat cell; and d) the ability to induce apoptosis in a Jurkat cell.

Brief Summary Text (25):
In another aspect, the invention provides a process for preparing a composition comprising a mixture of one or more isolated triterpene glycosides, comprising: a) obtaining tissue from an Acacia victoriae plant; b) extracting the tissue with a solvent to provide an extract; and c) obtaining one or more triterpene glycosides from the extract. The tissues used in this process typically comprises pods, roots, seedlings, or mixtures thereof. The solvent used for the extraction may be any organic solvent which is capable of extracting, often by dissolving, the saponin compound of interest. Useful extraction solvents are methanol, ethanol, isopropyl alcohol, dichloromethane, chloroform, ethyl acetate, water, glycerol and mixtures thereof.

Detailed Description Text (8):
An important aspect in the use of plant extracts as pharmaceutical preparations is the characterization and determination of the individual active constituents. Such also is the case for triterpene saponin preparations, which often require sophisticated techniques for the isolation, structure elucidation and analysis of their components and glycosides. When biological testing of the pure compounds is to be performed, it is necessary to isolate them in sufficient quantity and purity.

Detailed Description Text (9):
Since triterpenes and other related saponins have relatively large molecular weights and are of high polarity, their isolation can be challenging. A problem involved in the isolation of pure saponins is the presence of complex mixtures of closely related compounds, differing subtly either in the nature of the aglycone or the sugar part (nature, number, positions and chirality of attachment of the monosaccharides). Difficulties also are encountered with labile substituents such as esters. For example, the major genuine soybean saponin, a .gamma.-pyrone derivative (BOA), is only extracted by aqueous ethanol at room temperature. Extraction with heating (80.degree. C.) leads to fission of the ester moiety and formation of soyasaponin I (Bb) (Kudou et al., 1992). In plants, saponins are accompanied by very polar substances, such as saccharides and coloring matter, including phenolic compounds and the like, are not easily crystallized, and can be hygroscopic, making it even more difficult to obtain crystals.

Detailed Description Text (16):
(ii) Extraction and Preliminary Purification

Detailed Description Text (17):
Extraction procedures should be as mild as possible because certain saponins can undergo transformations including enzymatic hydrolysis during water extraction, esterification of acidic saponins during alcohol treatment, hydrolysis of labile ester groups and transacylation. Therefore, care should be taken to follow the individual steps in an isolation procedure, for example, in thin layer chromatography.

Detailed Description Text (18):
Although numerous variations are possible, current general procedures for obtaining crude saponin mixtures typically include extraction with methanol, ethanol, water or aqueous alcohol; a defatting step, generally with petroleum ether, performed before the extraction step or on the extract itself; dissolution or suspension of the extract in water; shaking or washing the solution or suspension with n-butanol saturated with water; and precipitation (optional) of saponins with diethyl ether or acetone. A dialysis step also can be included in order to remove small water-soluble molecules such as sugars (see, for example, Zhou et al., 1981; Massiot et al., 1988).

Detailed Description Text (19):
The most efficient extraction of dry plant material is achieved with methanol or aqueous methanol. Methanol is also used for fresh plant material. Although water is typically a less efficient extraction solvent for saponins (unless specifically water-soluble glycosides are desired) it has the advantages of being easily lyophilized and giving a cleaner extract. Depending on the proportion of water used for extraction, either monodesmosidic or bidesmosidic saponins may be obtained (Domon and Hostettmann, 1984; Kawamura et al., 1988). Fresh vegetable material contains active enzymes (esterases) which, when homogenized with a solvent, are able to convert bidesmosides into mono-desmosides. Even dry material may contain esterases which are activated in the presence of water. In the case of momordin I (a monodesmosidic oleanolic acid saponin) it was found that conversion to momordin II (the corresponding bidesmoside) takes place in water and in 30% and 60% methanol solutions, but not in 80% and 100% methanol solutions. On the contrary, homogenates of the fresh roots in methanol retained enzyme activity. However, the enzymes could be inactivated by first soaking the fresh roots in 4% hydrochloric acid and the bidesmoside was then shown to be the major component. It is, therefore, clear that the correct choice of extraction procedure is an extremely important first step.

Detailed Description Text (21):
A common problem observed for furostanol saponins is the formation of 22-OCH.sub.3 derivatives during extraction with methanol. However, the genuine 22-hydroxyfurostanols can either be obtained by extraction with another solvent (e.g., pyridine) or by treatment of the methoxylated artifacts with boiling aqueous acetone (Konishi and Shoji, 1979).

Detailed Description Text (23):
The qualitative analysis of triterpene saponins by TLC is of great importance for all aspects of saponin investigations. TLC plates (usually silica gel) can handle both pure saponins and crude extracts, are inexpensive, rapid to use and require no specialized equipment. A number of visualization reagents are available for spraying onto the plates (Table 2). Methods of preparation of the most common reagents are as follows:

Detailed Description Text (28):
Spraying with vanillin-sulfuric acid in the presence of ethanol and perchloric acid, for example, gives a blue or violet coloration with triterpene saponins. With anisaldehyde-sulfuric acid, a blue or violet-blue coloration is produced on heating the TLC plate. Spraying TLC plates with a solution of cerium sulphate in sulfuric acid gives violet-red, blue or green fluorescent zones under 365 nm UV light (Kitagawa et al., 1984b). In some cases, simply spraying the plates with water is sufficient to reveal the saponins present. Additional spray reagents may be found in, for example, Stahl (1969).

Detailed Description Text (81):
A variety of separation techniques have been described and may be used for separating triterpene saponins including flash chromatography, DCCC, low-pressure liquid chromatography (LPLC), medium-pressure liquid chromatography (MPLC), HPLC and conventional open-column chromatography (See, e.g., Hostettmann et al., 1986, 1991; Marston and Hostettmann, 1991 b). An idea of separation conditions, solvent systems, etc. will be known to those of skill in the art in light of the instant disclosure. The best results are usually achieved by strategies which employ a combination of methods, such as those specifically disclosed herein below.

Detailed Description Text (104):
For example, a combination of MPLC on silica gel and RP material, LPLC and centrifugal TLC for separation of saponins (Hamburger and Hostettmann, 1986). Similarly, the isolation of five triterpene saponins from Swartzia madagascariensis (Leguminosae) required open-column chromatography, LPLC and MPLC (Borel and

Hostettmann, 1987).

Detailed Description Text (107):
Another strategy involves passing extracts (after preliminary partition) over highly porous polymers and following this step by further fractionation of the crude saponin mixtures. This approach was used in the isolation of 3.beta.-hydroxyolean-12-en-28,29-dioic acid glycosides from Nothopanax delavayi (Araliaceae). A methanol extract of the leaves and stems was partitioned between hexane and water. The aqueous layer was chromatographed on a Diaion HP-20 column and eluted with water, 10% methanol, 50% methanol, 80% methanol, methanol and chloroform. The glycosides were obtained by subsequent column chromatography of the 80% methanol eluate on silica gel with ethyl acetate-ethanol-water (7:2:1) (Kasai et al., 1987a). For the isolation of triterpene and non-triterpene saponins from Acanthopanax senticosus (Araliaceae), the procedure began with a fractionation of the methanol extract of the leaves on Diaion HP-20 polymer. The fraction eluted with methanol was chromatographed on silica gel (chloroform-methanol-water 30:10:1) and all the resulting fractions were subjected to column chromatography on LiChroprep RP-8. Final purification was achieved by HPLC on TSK-GEL ODS-120T (300.times.21 min; methanol-water 70:30; 6 ml/min; RI detection) or chromatography on a hydroxyapatite column (acetonitrile-water 85:15) (Shao et al, 1988).

Detailed Description Text (110):
Reactions of triterpenes with any of a variety of agents may be used to produce colored compounds for the quantitative or qualitative determination of triterpenes. For example, aromatic aldehydes such as aisaldehyde and vanillin in strong mineral acid, for example, sulfuric, phosphoric, and perchloric acids, give colored products with aglycones, having absorption maxima between 510 and 620 nm. In these reactions, a dehydration is believed to occur, forming unsaturated methylene groups which give colored condensation products with the aldehydes. With vanillin-sulfuric acid, triterpene saponins with a C-23 hydroxyl group have a peak located between 460 and 485 nm (Hiai et al., 1976).

Detailed Description Text (115):
Legume extracts were prepared by chloroform:methanol or dichloromethane: chloroform extraction at The University of Arizona (Tucson, Ariz.). The inventors isolated mixtures of triterpene glycosides from Acacia victoriae (Benth.) (Leguminosae). The first collection of UA-BRF-004-DELEP-F001 was processed as follows: (1) grinding to 3 mm particle size in Wiley mill, (2) packing into two-liter percolation unit, (3) extracting the ground biomass with dichloromethane:methanol (1:1) for 4 hr. followed by overnight and the combined fractions were dried in vacuo to generate UA-BRF-004-DELEP-F001 (52 g). F001 (51.5 g) was extracted with ethyl acetate to yield active insoluble (34.7 g) material designated as F004. Flash chromatography using 1.7 kg of silica gel (Merck, 23-220 micron particle size) was used to fractionate F004 (34.2), 51 670-ml fractions eluted with dichloromethane: methanol (step-gradient-95-0%: methanol 5-100%). the Column was washed with nine-liters of methanol followed by six-liters of methanol:water (80:20) and then six-liters of same eluent with 1% formic acid added. Based on TLC fractions 23-34 and 39-40 were combined to 17.2 g of F023. Medium Pressure Liquid Chromatography (MPLC, Buchi 632 system) was used twice with 8 g of F023 each on a 4.9.times.46-cm column packed with Lichroprep C18, 15-25 micron particle size using step gradient of acetonitrile: water (0,10,20,30,50% acetonitrile in water) followed by 100% methanol wash. Of the 16 g 0-20% acetonitrile, yield was seven grams of F027, which was inactive. The remaining material was combined and subjected to repetitive MPLC with the same system using 30-40% acetonitrile to minimize overlap and generate fractions F028-F036. Although most of these fractions demonstrated antitumor activity, F035 (Fraction 35) (highest yield of 2.19 g) was selected for further testing and evaluation.

Detailed Description Text (117):
Various methods may be employed for the qualitative and quantitative determination

of triterpenes and their activities including: piscicidal activity, gravimetry, spectrophotometry, TLC, GC, HPLC, HMQC, HMBC, NOESY, COSY, NMR, X-Ray crystallography etc. Determinations based on classical properties of triterpene saponins (surface activity, fish toxicity) have largely been replaced by photometric methods such as densitometry, colorimetry of derivatives and, more recently, by GC, HPLC and particularly, NMR. Spectrophotometric methods are very sensitive but not typically suitable for estimating triterpenes in crude plant extracts since the reactions are not specific and colored products may form with compounds which accompany the triterpenes, such as phytosterols and flavonoids. Another problem, common to much of the analytical work on saponins, is their incomplete extraction from the plant material. However, a number of techniques are widely available which are suitable for quantitating triterpenes.

Detailed Description Text (125):
For assigning chemical shifts, it is helpful to compare observed data with data reported for model and related compounds. As a guide to some of the typical chemical shifts in the .sup.13 C-NMR spectrum of a triterpene saponin, one may use the known shifts of the bayogenin glycoside (Domon and Hostettmann, 1984). Additionally, compilations of assignments of .sup.13 C-NMR signals for oleanane (Patra et al., 1981; Agrawal and Jain, 1992), ursane, lupane (Wenkert et al., 1978; Sholichin et al., 1980), hopane (Wenkert et al., 1978; Wilkins et al., 1987) and lanostane (Parrilli et al., 1979) triterpenes have been made (Nakanishi et al., 1983). The relevant data for dammarane glycosides have been summarized in a review (Tanaka and Kasai, 1984), while .sup.13 C-NMR spectroscopy of saikogenins (Tori et al., 1976a) and of saikosaponins (Tori et al., 1976b) has been described. Ginseng sapogenins and related dammarane triterpenes also have been studied (Asakawa et al., 1977). .sup.13 C-NMR spectroscopy of acacic acid has also been described (Kinjo et al., 1992).

Detailed Description Text (134):
In practice, certain .sup.1 H and .sup.13 C NMR spectra can be identified and assigned on the basis of shift arguments, but for interpreting the results of NMR studies in a rigorous manner, an NMR spectrum should be assigned unambiguously, which means establishing which peaks are associated with which carbon and/or hydrogen in the structure. This information, in most cases, cannot be obtained from one-dimensional .sup.1 H and .sup.13 C NMR spectral data, but can better be determined with the aid of two-dimensional studies. These studies simplify spectral analysis by spreading out information into two frequency domains and by revealing interactions between nuclei. Despite the fact that the mechanisms on which the various pulse sequences are established may be intricate, the interpretation of two-dimensional NMR spectra is usually straightforward. A large number of different two-dimensional NMR studies have been devised to solve chemical structures. Examples of such techniques, as well as other NMR techniques specifically contemplated by the inventors for use in the chemical elucidation of the triterpene saponins of the invention, are described below, and in Table 3.

Detailed Description Text (144):
The sequences of sugar and interglycosidic linkages of triterpene glycosides from marine organisms have been established from NT.sub.1 data and NOESY studies (Miyamoto et al., 1990) but this methodology is limited by the complexity of the .sup.1 H-NMR spectra in the 3-5 p.p.m. region, which usually precludes the measurement of NOE for a large number of protons. However, a combination of COSY, NOESY and direct and XHCORR NMR spectroscopy has allowed complete signal assignment and structural analysis of pentasaccharide triterpene saponins from the sea cucumber Holothuria forskalii (Rodriguez et al., 1991).

Detailed Description Text (188):
Triterpene saponins are glycosides in which the hemiacetal hydroxyl groups of saccharides in their cyclic pyranose or furanose forms build acetals with a triterpene or steroid residue. The ether linkage between the hemiacetal hydroxyl

and the triterpene or steroid is known as a glycosidic linkage. The monosaccharide constituents of the oligosaccharides also are bound by ether linkages (interglycosidic bonds).

Detailed Description Text (190):
Numerous chemical reactions and methods have been employed for breaking down saponins into smaller units for more ready analysis (see, for example, Kitagawa, 1981). Such methods will find particular use in structural determinations of triterpene saponins.

Detailed Description Text (192):
Acidic hydrolysis maybe carried out by refluxing the saponin in acid for a fixed length of time, for example, 4 h in 2-4 M hydrochloric acid. The aqueous solution remaining after hydrolysis is extracted with diethyl ether, chloroform or ethyl acetate to obtain the aglycone. Extraction of the sugars from the aqueous layer is performed with pyridine, after neutralizing the solution (with alkali or basic ion exchange resin) (Tschesche and Forstmann, 1957; Sandberg and Michel, 1962) and evaporation to dryness. The saponins are completely cleaved into their constituents by this method so information is obtained as to the identity of the aglycone and the number and nature of monosaccharides present. If a prosapogenin (obtained after cleavage of an ester linkage by basic hydrolysis) is acid hydrolyzed, the nature of the sugar chains which are ether-linked to the aglycone can be established. An aqueous reaction medium can be replaced by alcohol or dioxane.

Detailed Description Text (412):
Sixty plant species were chosen from the Desert Legume Project (DELEP) with the goal of identifying novel compounds having beneficial biological activities. The DELEP (University to of Arizona, Tucson) is a collection of desert legume species developed through a collaboration between the University of Arizona and the Boyce Thompson Southwestern Arboretum. Experimental field samples were collected from each of the plant species, air-dried for 3-4 days, ground to three millimeter particle size with a Wiley mill (3 mm screen size) and extracted two or three times by percolation with a 1:1 mixture of dichloromethane (DCM) and methanol (MeOH). Each percolation extraction proceeded for at least 5 hours and often continued overnight. The majority of the extracted biomass was collected from the first two percolations. The biomass was then washed with a volume of methanol equal to half the void volume, and the crude extract contained in the methanol aliquots isolated. The samples were typically isolated and prepared for bioassay by removing the methanol in vacuo, passing the aqueous phase through RP-C 18 particles, recovering the active constituents in MeOH, and then rotovapping the MeOH to collect the extract as a solid. The crude extract was then resuspended in H20, DMSO or mixtures thereof (less polar compounds were resuspended in DMSO, while more polar compounds were resuspended in water or water and DMSO mixtures; aglycones were resuspended in DMSO).

Detailed Description Text (430):
Although the above procedures focused on the isolation of active constituents from pods of Acacia victoriae, the active constituents may also be extracted from roots. In this case, the roots are ground for 1/2 hour and covered with 100% MeOH. The mixture is then filtered and diluted to 80% MeOH in water. If large amounts of roots are to be extracted, then it may be preferable to extract via percolation as described above. The reason for the differences in these extraction procedures is that roots are typically extracted fresh while the pods are often dried prior to extraction.

Detailed Description Text (433):
A modified extraction/separation procedure was used for the scaled-up preparation of mixtures of active constituents from fraction UA-BRF-004Pod-DELEP-F094 (F094). This procedure was repeated multiple times, consistently yielding highly active fractions. Typically, 20-25 g of F094 or its equivalent was dissolved in 150-175 ml

of 50% MeOH in H.sub.2 O which was then aspirated onto a column ((26 mm.times.460 mm)+(70 mm.times.460 mm), RP-C18, 40 .mu.m, 1200 g, equilibrated with 60% MeOH/H.sub.2 O). The fractions were eluted in steps of 8 L in 60% MeOH/H.sub.2 O; 7.5 L 70% MeOH/H.sub.2 O; and 2 L MeOH and assigned fraction identifiers as shown in Table 15. Fraction F035-B2 contains a mixture of the active components contained in F094, F133-136 (isolated from F093) and F138-147 (isolated from F094) as shown in FIGS. 18A-18F. F094 is an acceptable substitute for F035 with a one- to two-fold decrease in potency and F035-B2 has less potency than F094.

Detailed Description Text (435):
Further improvements to the above extraction procedure, as well as the other extraction procedures disclosed herein, may be realized by using tri-solvent mixtures of acetonitrile, methanol and water. The percentage ranges can be dynamically produced and optimized by anyone familiar with standard chromatographic techniques. Likewise, bonded phase silicas can be varied by using a combination of RP systems, including, but not limited to C-8, CN, dimethyl diol and C-18. In the final steps, even normal phase silica can be utilized for final purification procedures.

Detailed Description Text (533):
The isolation of B1 was accomplished by plant extraction and C-18 flash chromatography followed by C-18 prep and semi-prep chromatography. The NMR of B1 indicates the same triterpene/monoterpene/quinovose/monoterpene structure as has been seen throughout this saponin family. The NMR also indicates the presence of four deoxy sugars and one N-acetyl group, which indicates that this molecule must differ from D1 in its sugar portions. See Table 21 for specific .sup.13 C-NMR assignments under (21). This molecule was degraded as shown in FIG. 38.

Detailed Description Text (571):
Next, in order to further study the mechanism by which the active components inhibited tumor cells, the TNF-alpha induced activation of the transcription factor NF-.kappa.B was analyzed in Jurkat cells (3.times.10.sup.6) which had been treated with 1-2 .mu.g/ml of UA-BRF-004-DELEP-F035 and UA-BRF-004Pod-DELEP-F094. The study was carried out as follows: Jurkat cells were pretreated with 1-2 .mu.g/ml of F035 or F094 for 15 h at 37.degree. C. Cells were harvested and resuspended in 1 ml RPMI and treated with 100 pM of TNF-alpha for 30 min at 37.degree. C. After TNF-alpha treatment, nuclear extracts were prepared according to Schreiber et al. (1989). Briefly, the cells were washed with ice cold PBS and suspended in 0.4 ml of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, 2 .mu.g/ml of leupeptin, 2 .mu.g/ml of aprotinin and 0.5 mg/ml benzamidine). The cells were allowed to sit on ice for 15 min and 25 .mu.l of 10% Nonidet-40 was added to the cells. The tubes were mixed on the vortex and microcentrifuged for 30 s. The nuclear pellet was resuspended in 25 .mu.l of ice cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 2 .mu.g/ml leupeptin, 2.0 .mu.g/ml aprotinin and 0.5 mg/ml benzamidine) and tubes were incubated on ice with intermittent agitation. The nuclear extract was microcentrifuged for 5 runs at 4.degree. C. and supernatants were stored at -70.degree. C.

Detailed Description Text (692):
Caspase-3 activity was measured as described earlier (Enari et al., 1995) with some modifications. Briefly, Jurkat cells (1.times.10.sup.6 /ml) were treated with F035, D1 & G1 for different lengths of time. Cytosolic extracts were prepared by repeated freeze thawing in 300 .mu.l of extraction buffer (12.5 mM Tris, pH 7.0, 1 mM DTT, 0.125 mM EDTA, 5% glycerol, 1 .mu.M PMSF, 1 .mu.g/ml leupeptin, 1 .mu.g/ml pepstatin and 1 .mu.g/ml aprotinin). Cell lystates were diluted 1:2 with ICE buffer (50 mM Tris, pH 7.0, 0.5 mM EDTA, 4 mM DTT and 20% glycerol) and incubated with 20 .mu.M of a caspase 3 substrate (acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin) at 37.degree. C. Caspase-3 activity was monitored by the production of fluorescent aminomethylcoumarin, which was measured at excitation 355 nM, emission 460 nM using

Fluoroscan II (Labsystems, Helsinki, Finland).

Detailed Description Text (716):
For harvesting of roots, the root mass of a single plant is rinsed with water directly in the aeroponic box and the root mass is cut with scissors a few inches above the sprayer. The excess water is removed by patting dry with paper towels, followed by weighing of the sample. The root mass is then cut in 3-4 inch sections with scissors and subject to chemical extraction, as described above. Alternatively, for continual harvest of roots, the pump is turned off and roots are clipped from the growing root mass. These roots are then cut into 3-4 inch sections and extracted as described. Care is taken not to damage the non-harvested roots.

Detailed Description Text (754):
Different media were tested for growth of hairy roots. Best growth was obtained on MS medium containing 2% sucrose. The effect of different capacity flasks and gibberellic acid was tested on the growth of hairy roots. The hairy roots were also grown on MS liquid medium on gyratory shaker in a 125 ml conical flask with 20 ml medium. An increase in growth of 84 fold was noted in 4 weeks. The production of triterpene saponins corresponding to those identified in F035 was confirmed by HPLC analysis with a standard authentic sample.

Detailed Description Paragraph Table (1):
TABLE 1 Applications of MPLC in the Separation of Triterpene Saponins Plant Support Solvent Reference Cussonia spicata Silica gel CHCl.sub.3 --MeOH--H.sub.2 O Gunzinger et al, 1986 (6:4:1) C-8 MeOH--H.sub.2 O (2:1) Gunzinger et al, 1986 Calendula arvensis C-8 MeOH--H.sub.2 O (65:35, Chemli et al., 1987 73:27) C. officinalis Silica gel CHCl.sub.3 MeOH H.sub.2 O Vidal-Ollivier et al., (61:32:5) 1989 C-18 MeOH-H.sub.2 O (60:40, Vidal-Ollivier et al., 80:20) 1989 Polygala Silica gel CH.sub.2 Cl.sub.2 --MeOH H.sub.2 O Hamburger and chamaebuxus (80:20:2) Hostettmann, 1986 C-8 MeOH--H.sub.2 O (55:45) Hamburger and Hostettmann, 1986 Swartzia C-8 MeOH H.sub.2 O (65:35) Borel and Hostettmann madagascariensis 1987 Talinum C-8 MeOH--H.sub.2 O (60:40) Gafner et al., 1985 tenuissimum Sesbania sesban C-8 MeOH--H.sub.2 O (55:45, Dorsaz et al, 1988 60:40) Tetrapleura C-8 MeOH--H.sub.2 O (70:30) Maillard et al., 1989 tetraptera Albizzia lucida C-8 MeOH--H.sub.2 O (6:4 .fwdarw. 9:1) Orsini et al., 1991 C-18 MeOH--H.sub.2 O (7:3) Orsini et al., 1991 Passiflora C-18 MeOH--H.sub.2 O (17:3) Orsini and Verotta, quadrangularis 1985 Hedera helix C-18 MeOH--H.sub.2 O gradient Elias et al., 1991 Primula veris C-18 MeOH--H.sub.2 O (5:5 .fwdarw. 7:3) Calis et al., 1992 Silica gel CHCl.sub.3 --MeOH--H.sub.2 O (61:3 Calis et al., 1992 2:7) Steroid saponins Balanites Silica gel CHCl.sub.3 --MeOH--H.sub.2 O Hosny et al., 1992 aegyptiaca (80:20:1 .fwdarw. 25:25:2 and 70:30:3)

Detailed Description Paragraph Table (2):
TABLE 2 Visualization Reagents for Triterpene Saponins Reagent Reference Vanillin-sulfuric acid Godin, 1954 Vanillin-phosphoric acid Oakenfull, 1981 Liebermann-Burchard (acetic Abisch and Reichstein, 1960 anhydride-sulfuric acid) Wagner et al, 1984 1% Cerium sulphate in 10% Kitagawa et al, 1984b sulfuric acid 10% Sulfuric acid in ethanol Price et al., 1987 50% Sulfuric acid Price et al, 1987 p-Anisaldehyde-sulfuric acid Wagner et al., 1984 Komarowsky Wagner et al., 1985 (p-hydroxybenzaldehyde-sulfuric acid) Antimony (III) chloride Wagner et al., 1984 Blood Wagner et al., 1984 Water

Detailed Description Paragraph Table (3):
TABLE 3 Selected NMR Approaches for Use in the Structure Establishment of Triterpene Saponins NMR Study (Acronyms) Comments Attached proton test (APT), Distortionless Discriminates among carbon types; enhancement by polarization transfer (DEPT), Spectral editing Insensitive nuclei enhancement by polarization transfer (INEPT) Incredible natural abundance double-quantum .sup.13 C--.sup.13 C connectivity, establishment transfer study (INADEQUATE) of molecular skeleton .sup.1 H, .sup.1 H-COSY Homonuclear shift correlation a) normal

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	DB=PGPB,USPT,USOC,EPAB,JPAB,DWPI; PLUR=YES; OP=ADJ		
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☐	L2	triterpene	2467
☐	L3	L2 near l1	181
☐	L4	l3 and extraction	82
☐	L5	L3 near isolation	3
☐	L6	maesa balansae	3
☐	L7	l3 and MW 1532	2
☐	L8	myrsinaceae	40
☐	L9	L8 and l3	3

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